UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR
CENTRO DE BIOTECNOLOGIA

Relação entre genes plasmidiais e virulência e análise do Sistema de Secreção Tipo 6 em isolados de *Escherichia coli* patogênica aviária (APEC)

Dissertação de Mestrado

ALINE LUÍSA DE OLIVEIRA

Porto Alegre, 2015

UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
PROGRAMA DE PÓS-GRADUAÇÃO EM BIOLOGIA CELULAR E MOLECULAR
CENTRO DE BIOTECNOLOGIA

Relação entre genes plasmidiais e virulência e análise do Sistema de Secreção Tipo 6 em isolados de *Escherichia coli* patogênica aviária (APEC)

ALINE LUÍSA DE OLIVEIRA

Orientação: Prof[a] Dr[a] Fabiana Horn

Trabalho apresentado como pré-requisito para obtenção do título de
Mestre em Biologia Celular e Molecular

Porto Alegre, 2015

Este trabalho foi desenvolvido no Laboratório de Microbiologia Celular, situado no Departamento de Biofísica da Universidade Federal do Rio Grande do Sul (UFRGS).

RESUMO

Escherichia coli patogênicas aviárias (APEC) causam infecções extraintestinais em aves, que podem ser localizadas ou sistêmicas, denominadas colibacilose. O patotipo de APEC não está definido, mas vários genes de virulência são associados a essas cepas, como os genes plasmidiais *iroN*, *ompT*, *hlyF*, *iss* e *iutA*, propostos, em 2008, como preditores da virulência de APEC. Além dos genes de virulência conhecidos, outros fatores podem estar associados à patogenicidade bacteriana, como as maquinarias de secreção protéica denominadas Sistemas de Secreção. O Sistema de Secreção do Tipo 6 (T6SS), descrito em 2006, tem sido associado à virulência de cepas APEC. Este trabalho divide-se em duas partes: a primeira teve como objetivo avaliar a frequência dos genes *iroN*, *ompT*, *hlyF*, *iss* e *iutA* em 401 cepas aviárias de *E. coli e* sua relação com a patogenicidade *in vivo* dessas cepas. A segunda parte teve como objetivos verificar a frequência de genes componentes do T6SS (*clpV*, *vgrG*, *icmF* e *dotU*), em uma coleção de 187 cepas APEC; e, em algumas cepas positivas para os genes, verificar a expressão de um fenótipo relacionado ao sistema, bem como a expressão do efetor *vgrG* e da ATPase do sistema *clpV2*, além da secreção de proteínas, no meio de cultura e durante contato com células eucarióticas. Os resultados da primeira parte indicam que cepas com dois ou mais dos genes analisados tem maior probabilidade de serem patogênicas do que cepas com apenas um ou nenhum dos genes. Já os da segunda parte mostram que várias cepas apresentaram duas cópias de pelo menos um dos genes testados, e algumas delas apresentaram resistência à predação por *D. discoideum*. Não foram encontradas diferenças entre a expressão dos genes *vgrG* (1 e 2) e *clpV2* em cultura pura ou em contato com células eucarióticas. Este trabalho apresenta a triagem de genes plasmidiais em uma grande coleção de amostras de *Escherichia coli*, e a primeira triagem de genes do T6SS em uma coleção de isolados APEC.

Palavras-chave: APEC, colibacilose, genes plasmidiais, T6SS, patogenicidade.

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) causes extraintestinal infections in birds, which can be localized or systemic, known as colibacillosis. The APEC pathotype is still undefined, but several virulence genes are associated with these strains, as the plasmid-linked genes *iroN*, *ompT*, *hlyF*, *iss* and *iutA*, proposed in 2008 as APEC virulence predictors. Besides the known virulence genes, other factors may be associated with bacterial pathogenicity, such as the protein secretion machineries called Secretion Systems. Described in 2006, Type 6 Secretion System (T6SS) has been associated with virulence of APEC strains. This work is divided in two parts: the first aimed to evaluate the frequency of *iroN*, *ompT*, *hlyF*, *iss* and *iutA* genes in 401 avian strains of *E. coli* and its relationship with *in vivo* pathogenicity of these strains. The second part aimed to verify the frequency of T6SS genes (*clpV*, *vgrG*, *icmF* and *dotU*) in a collection of 187 APEC strains; and verify, in some positive strains, the expression of a phenotype related to the system, as well as the gene expression of effector *vgrG* and ATPase *clpV2*, besides the secretion of proteins into the culture medium and during contact with eukaryotic cells. The results of the first part of this study indicate that isolates harboring two or more of the genes analyzed were most likely to be pathogenic than strains harboring only one or none of the genes. The results of the second part show that several strains harbored two copies of at least one of the genes tested, and some of them were resistant to predation by *D. discoideum*. No differences were found between the expression of genes *vgrG* (1 and 2) and *clpV2* in pure culture or in contact with eukaryotic cells. This work presents the screening of plasmidial genes in a large collection of *Escherichia coli* isolates, and the first screening of T6SS genes in a collection of APEC isolates.

Keywords: APEC, colibacillosis, plasmid-linked genes, T6SS, pathogenicity.

SUMÁRIO

1. INTRODUÇÃO

1.1. *Escherichia coli*

Escherichia coli é um bacilo gram-negativo, anaeróbio facultativo, que apresenta distribuição cosmopolita e coloniza o intestino de humanos e animais endotérmicos logo após o nascimento. *Escherichia coli* se estabelece como um importante membro da microbiota intestinal, raramente causando doença, exceto em caso de imunodepressão ou do rompimento das barreiras gastrintestinais (Kaper *et al.*, 2004). Devido a isso, *E. coli* foi por muito tempo considerada uma bactéria não-patogênica, responsável por auxiliar na prevenção da colonização do epitélio intestinal por microrganismos patogênicos. No entanto, a aquisição de fatores de virulência por algumas linhagens de *E. coli* tornou-as patogênicas, ou seja, capazes de causar diversas doenças em humanos e outros animais (Barnes e Gross, 1997; Dho-Moulin e Fairbrother, 1999; Ferreira e Knöbl, 2000; Kaper *et al.*, 2004).

1.2. *Escherichia coli* patogênica aviária (APEC)

Escherichia coli patogênicas são classificadas em dois grupos: (1) InPEC (Intestinal Pathogenic *E. coli*), responsáveis pelas diarreias, e que podem apresentar diversas estratégias para invadir as células intestinais (Reis e Horn, 2010); e (2) ExPEC (Extraintestinal Pathogenic *E. coli*). Cepas ExPEC possuem fatores de virulência que as conferem a capacidade de viver fora do intestino e as distinguem de cepas comensais e patogênicas intestinais, tornando-as capazes de causar várias doenças em humanos e outros animais (Russo e Johnson, 2000). Devido a sua diversidade, o grupo ExPEC é dividido em subpatotipos, incluindo UPEC (Uropathogenic *E. coli*), causadoras de infecção do trato urinário; NMEC (Newborn Meningitis-causing *E. coli*), causadoras de meningite neonatal; e APEC (Avian Pathogenic *E. coli*), causadoras da colibacilose em aves (Kaper *et al.*, 2004; Dobrindt, 2005).

1.3. Colibacilose aviária

A colibacilose aviária, doença que se manifesta como infecções localizadas ou sistêmicas (colissepticemia), é mais comum em frangos, mas pode também ocorrer em outras espécies de aves domésticas e silvestres (Barnes e Gross, 1997; Dho-Moulin e Fairbrother, 1999; Barbieri *et al.*, 2012). A colissepticemia, forma mais grave da doença, geralmente inicia-se no trato respiratório superior e pode instalar-se em diferentes locais anatômicos da ave e apresentar sintomatologia variada (Ngeleka *et al.*, 1996; Dho-Moulin e Fairbrother, 1999).

Diversos fatores ambientais podem tornar as aves mais suscetíveis à infecção por APEC, por oferecerem à bactéria um ambiente mais propício ao seu desenvolvimento. Entre eles estão: a alta concentração de amônia no ambiente (que pode causar danos no epitélio ciliar do trato respiratório, facilitando a entrada da bactéria); a deficiência no sistema de ventilação, a exposição a temperaturas extremas, a limitação do espaço para a criação e a deficiência do processo de desinfecção (Dho-Moulin e Fairbrother, 1999; Ferreira e Knöbl, 2000). Por afetar todas as etapas da produção avícola, a colibacilose contribui para a diminuição da qualidade da produção, constituindo-se em um problema para a economia do Brasil, que é o maior exportador mundial e terceiro maior produtor de carne de frango (Ubabef, 2014).

1.4. Fatores de virulência

Vários fatores de virulência, como adesinas, invasinas, fatores de resistência ao soro, sistemas de aquisição de ferro e toxinas já foram associados à patogenicidade de cepas APEC (Barbieri *et al.*, 2013; Barbieri *et al.*, 2014). Esses fatores podem ser encontrados em elementos genéticos, como plasmídeos, transposons, ilhas de patogenicidade e bacteriófagos (Falkow, 1991). Grandes plasmídios, como o ColV, tem sido associados à virulência de *E. coli* (Waters e Crosa, 1991), sendo comuns em cepas APEC (Johnson *et al.*, 2006) e

reconhecidos pela importante contribuição para esse patotipo (Ginns *et al.*, 2000; Johnson *et al.*, 2002; Rodriguez-Siek *et al.*, 2005; Johnson *et al.*, 2006).

1.5. Genes plasmidiais

Em 2005, foi sequenciado o primeiro plasmídeo ColV, o pAPEC-O2-ColV, derivado da cepa APEC O2 (O2:K2) isolada da articulação de um frango com colibacilose (Johnson *et al.*, 2002). Foi encontrada uma região de 93 kb contendo genes de virulência conhecidos ou putativos que explicariam a relação de tais plasmídeos com a virulência de APEC. Estão presentes nessa região fatores associados à virulência de APEC, como *iss*, *tsh*, o operon da salmoquelina e outros três sistemas de transporte e aquisição de ferro (*sitABC*, *eitABC* e *etsABC*), além de genes cuja função ainda não foi determinada, como *hlyF* e *ompT* e outros que, por análise *in silico*, codificam proteínas hipotéticas (Johnson *et al.*, 2006).

Em 2008, Johnson et al., demonstraram que os genes plasmidiais *iutA*, *iroN* (que codificam receptores sideróforos), *iss* (que codifica proteína de resistência ao sistema complemento), *ompT* (que codifica proteína de membrana externa) e *hlyF* (que codifica uma hemolisina putativa de *E. coli* aviária) eram significativamente mais frequentes em cepas APEC do que em cepas fecais de animais saudáveis, sugerindo que estes poderiam servir como indicadores da patogenicidade de APEC (Johnson *et al.*, 2008). Apesar da existência de fatores reconhecidos por sua contribuição para a virulência de APEC, ainda não há um marcador definido ou conjunto de marcadores que definam o patotipo APEC (Dziva e Stevens, 2008).

1.6. Sistemas de Secreção

A patogenicidade de uma bactéria pode também ser influenciada por maquinarias proteicas denominadas Sistemas de Secreção (Kostakioti *et al.*, 2005). Esses sistemas apresentam componentes que medeiam o transporte de proteínas efetoras através da membrana interna, do espaço periplasmático e da membrana externa da bactéria (Shrivastava e Mande, 2008). A secreção de

proteínas desempenha um papel central na modulação de diversas associações bacterianas, da formação de biofilmes a associações mutualísticas ou patogênicas com organismos hospedeiros (Tseng *et al.*, 2009). Dessa forma, a elucidação dos mecanismos utilizados pelas bactérias para a secreção de proteínas é importante para a compreensão de tais associações (Shrivastava e Mande, 2008).

Seis Sistemas de Secreção já foram descritos para bactérias Gram-negativas. Os sistemas 2 e 5 dependem dos sistemas de secreção universais Sec e Tat (*two-arginine*). Proteínas exportadas ao espaço periplasmático pelos sistemas universais são, então, transladadas através da membrana externa da bactéria pelos sistemas 2 e 5. Já os sistemas 1, 3, 4 e 6 secretam proteínas bacterianas através da membrana interna e externa da bactéria em uma única etapa (Tseng *et al.*, 2009).

Em bactérias Gram-positivas, o transporte de proteínas geralmente se dá através dos sistemas universais (Tseng *et al.*, 2009), exceto em casos específicos, como o da *Mycobacterium* que, devido à hidrofobicidade e alta impermeabilidade da membrana, desenvolveu um sistema especializado, denominado Tipo 7 (Abdallah *et al.*, 2007). Recentemente, foi proposto que a rota de nucleação-precipitação extracelular (ENP) envolvida na montagem da adesina curli fosse classificada como Sistema de Secreção Tipo 8 (Desvaux *et al.*, 2009).

1.7. Sistema de Secreção Tipo 6

O Sistema de Secreção Tipo 6, do qual trata este estudo, foi descrito em 2006 na cepa de *Vibrio cholerae* V52, responsável por um surto de cólera ocorrido no Sudão, em 1968. Diferentemente das cepas conhecidas por causarem pandemias, que geralmente pertencem aos sorogrupos O1 e O139, *V. cholerae* V52 pertence ao sorogrupo O37. Utilizando a ameba *Dyctiostelium discoideum* como modelo de hospedeiro, a fim de identificar mecanismos de virulência, os autores verificaram que a cepa V52 apresentou resistência à predação pela ameba, fenótipo não encontrado em outras linhagens de *V. cholerae*. A partir de uma triagem genética, os autores descobriram que tal fenótipo era dependente de

um sistema de secreção desconhecido, então denominado de Sistema de Secreção Tipo 6 (T6SS) (Pukatzki *et al.*, 2006).

O T6SS apresenta um operon composto por 15 a 20 genes, e, a partir da análise de genomas bacterianos, foi identificado um grupo de 13 proteínas conservadas que constitui o núcleo do T6SS (Shrivastava e Mande, 2008), e um conjunto de proteínas não conservadas, responsáveis por funções regulatórias e acessórias (Bingle *et al.*, 2008).

Diversos estudos acerca do T6SS já foram realizados, mas as funções dos genes que compõem este sistema ainda não foram elucidadas. Acredita-se que as proteínas que não são secretadas podem compor estruturalmente o aparato de secreção e/ou auxiliar no transporte de proteínas de alguma outra forma, atuando, por exemplo, no fornecimento da energia necessária ao transporte dos substratos pelo sistema (Filloux *et al.*, 2008). Embora a maioria dos componentes do T6SS não tenha sido caracterizada, alguns genes já foram descritos e tiveram sua função definida. É o caso dos genes *vgrG*, *clpV*, *icmF* e *dotU*, analisados neste trabalho.

A proteína VgrG (*valine-glycine repeat protein*) forma uma estrutura pontiaguda localizada na extremidade do sistema denominada “dispositivo de punção”, que é responsável por injetar as proteínas secretadas diretamente no interior da célula do hospedeiro (Bingle *et al.*, 2008; Cascales, 2008; Filloux *et al.*, 2008). Além disso, as proteínas VgrG de *V. cholerae* apresentam similaridade de sequência com as proteínas do injectissoma do bacteriófago T4, apresentando um domínio responsável por induzir ligações cruzadas de actina (Pukatzki *et al.*, 2007).

O gene *clpV* (*caseinolytic protease V*) codifica a ATPase ClpV. No primeiro modelo proposto para o sistema, ClpV foi descrita como responsável pelo fornecimento da energia necessária para a montagem e ativação do sistema (Bingle *et al.*, 2008; Cascales, 2008). No entanto, em um modelo mais recente, ClpV foi considerada responsável pela desmontagem de uma bainha de proteínas contráteis - responsáveis pela ativação do sistema - e reciclagem destas proteínas (Zoued *et al.*, 2014).

Os genes *icmF* e *dotU* foram descritos em *Legionella pneumophila* e pertencem, respectivamente, a conjuntos de genes denominados *icm* (intracellular multiplication) e *dot* (defect in organelle trafficking), que codificam o sistema de Secreção Tipo 4 (T4SS). São responsáveis por mediar a alteração da rota endocítica, permitindo a sobrevivência da bactéria no interior dos macrófagos (Segal *et al.*, 1998; Vogel *et al.*, 1998).

Através de análises genômicas, o sistema foi identificado em 42 espécies de bactérias patogênicas da divisão Proteobacteria (Shrivastava e Mande, 2008), e está amplamente distribuído nessa divisão, quase exclusivamente nos subgrupos α-,β- e γ-proteobacteria, sendo ainda mais frequente no último subgrupo (Bingle *et al.*, 2008; Boyer *et al.*, 2009). Análises *in silico* já detectaram 176 loci do T6SS em 92 espécies bacterianas (Boyer *et al.*, 2009).

1.8. T6SS e associações bacterianas

Estudos tem demonstrado a importância do sistema na patogênese de diversas espécies bacterianas. Em *Burkholderia mallei*, patogênica para humanos e outros animais, o T6SS é importante para a proliferação no interior de macrófagos, e uma proteína relacionada a Hcp (Hemolysin-coregulated protein, uma proteína efetora e estrutural do sistema) é induzida durante a infecção *in vivo* (Schell *et al.*, 2007). *Edwardsiella tarda*, patógeno de peixes, apresenta um T6SS ativo (Zheng e Leung, 2007), e, em *Pseudomonas aeruginosa*, Hcp1 é secretada por isolados clínicos, e pacientes com fibrose cística produzem anticorpos anti-Hcp1, indicando que o sistema está ativo durante a infecção (Mougous *et al.*, 2006).

Além da patogenicidade, o T6SS já foi relacionado a outras propriedades bacterianas. Estudos já demonstraram o envolvimento do T6SS na propriedade antibacteriana de *V. cholerae* em relação a outros patógenos gram-negativos, como *Salmonella Typhimurium*, *Citrobacter rodentium* e *Pseudomonas aeruginosa* (Macintyre *et al.*, 2010); e no favorecimento de *Pseudomonas fluorescens* na

competição com *Pectobacterium atrosepticum*, um patógeno vegetal, que causa o apodrecimento de tubérculos (Decoin *et al.*, 2014).

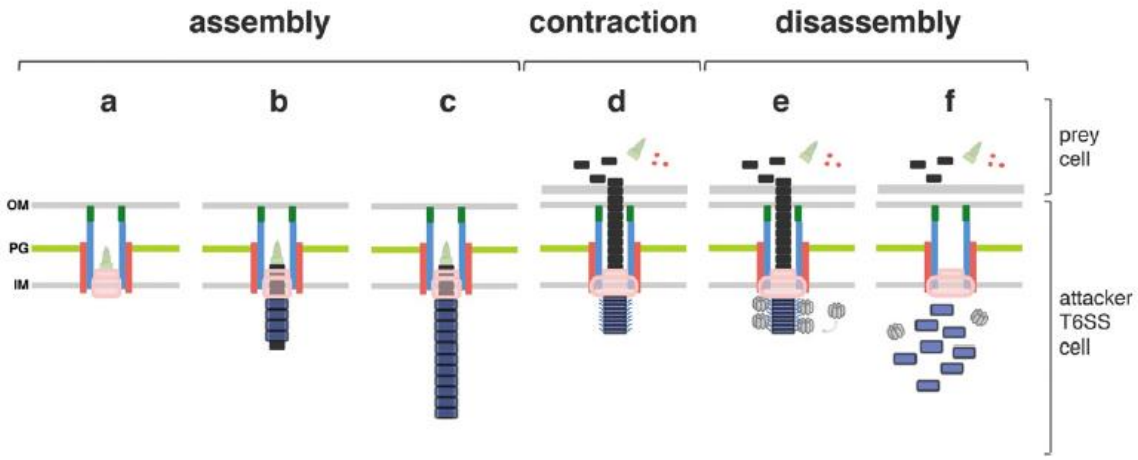
1.9. T6SS em APEC

A relação entre o T6SS e a virulência de APEC foi recentemente estabelecida. Em 2010, Pace *et al*. verificaram que a cepa APEC SEPT 362 expressa um Sistema de Secreção Tipo 6, e, através da geração de mutantes para genes do sistema (*hcp* e *clpV*), mostraram que o T6SS contribui para a virulência *in vivo* da cepa. Além disso, mutantes para *hcp* e *clpV* tiveram suas capacidades de adesão e de rearranjo do citoesqueleto de células epiteliais diminuídas, e o mutante *hcp* apresentou pequena diminuição na capacidade de invasão de células epiteliais. O comprometimento dessas características, de acordo com os autores do trabalho, pode ser devido à diminuição da expressão da fímbria do tipo 1 observada nesses mutantes (De Pace, F. *et al.*, 2010).

Já em 2011, o mesmo grupo demonstrou a relação de outro gene do sistema, *icmF*, com a patogênese da cepa SEPT 362. O mutante para *icmF*, apesar de não ter atenuado a virulência *in vivo*, também apresentou diminuição da adesão a e invasão de células epiteliais. Porém, nesse caso, o mutante não apresentou diminuição na expressão de *fimA,* sugerindo que a contribuição de *icmF* para a adesão é independente da fímbria. Além disso, o mutante *icmF* apresentou menor capacidade de formação de biofilmes e menor viabilidade no interior de macrófagos (De Pace *et al.*, 2011).

O interesse pelo T6SS em APEC tem aumentado nos últimos anos. Em 2013, três *loci* do T6SS foram encontrados na cepa APEC ED205, isolada de pato com colissepticemia. Foi realizada uma triagem desses *loci* em 472 isolados APEC, das quais 11 apresentaram dois *loci* do sistema. O *locus* 1, além de maior e mais frequente nas cepas analisadas, apresentou 15 proteínas conservadas do sistema. A diferença no número de genes conservados e na frequência dos diferentes *loci* sugere que eles possam desempenhar diferentes funções através de diferentes mecanismos (Ma *et al.*, 2013).

Já em 2014, dois trabalhos demonstraram a relação entre o T6SS e a patogenicidade de cepas APEC. O primeiro demonstrou que a cepa APEC TW-XM, isolada do cérebro de pato com septicemia e sinais neurológicos, apresenta dois T6SS funcionais (T6SS1 e T6SS2), sendo o T6SS1 associado à infecção sistêmica, e o T6SS2, com a infecção cerebral (Ma *et al.*, 2014). Ainda em 2014, foi demonstrado que a proteína DotU, codificada pelo *locus* do T6SS2 da cepa *E. coli* ED719, está relacionada à patogênese dessa cepa, à secreção da proteína Hcp1 e à modulação da resposta imunológica durante a infecção (Wang *et al.*, 2014).



Mecanismo de ação do T6SS: a) formação dos complexos de membrane e da base no local da secreção; b, c) alongamento da estrutura tubular citoplasmática formada por hexâmeros de Hcp (retângulos pretos) empilhadas uma na outra acoplados à polimerização da cauda de TssBC (retângulos azuis). Uma vez em contato com a célula-alvo: d) a cauda de TssBC contrai impulsionando o tubo interno em direção à célula-alvo para entregar as proteínas efetoras; and e) ATPase ClpV é recrutada para desmontagem do sistema e reciclagem das proteínas da cauda TssBC (Adaptado de Zoued *et. al.*, 2014).

2. OBJETIVOS

Frente ao exposto, este trabalho teve como objetivos:

Verificar a frequência dos genes plasmidiais *iroN*, *ompT*, *hlyF*, *iss* e *iutA* em cepas aviárias de *Escherichia coli* (APEC, de cama de aviário e fecais) e sua relação com a patogenicidade de cepas APEC;

Verificar a presença de genes do Sistema de Secreção Tipo 6, bem como a expressão desses genes e a funcionalidade do T6SS em cepas APEC.

2.1 Objetivos específicos

1. Detectar a presença dos genes *iroN*, *ompT*, *hlyF*, *iss* e *iutA*, por PCR, em cepas aviárias de *Escherichia coli* (APEC, de cama de aviário e fecais) ;
2. Realizar a tipagem filogenética das cepas de cama de aviário e fecais;
3. Avaliar a virulência em modelo *in vivo* das cepas de *E. coli* de cama de aviário;
4. Verificar se há relação da presença dos genes analisados com o índice de patogenicidade das cepas APEC (previamente estabelecidos) e de cama de aviário;
5. Detectar a presença dos genes componentes do Sistema de Secreção Tipo 6 (T6SS) *clpV*, *vgrG*, *icmF* e *dotU* em uma coleção de 187 isolados APEC;
6. Analisar o fenótipo característico do T6SS (resistência à predação pela ameba *Dyctiostelium discoideum*) em algumas cepas positivas para os genes analisados;
7. Avaliar a expressão de duas cópias do gene *vgrG* - que codifica uma proteína efetora do sistema - e do gene *clpV* (uma cópia) - que codifica uma ATPase - por diferentes cepas, incluindo APEC O1, quando em cultura pura e em contato com macrófagos e células não-fagocitárias.

3. RESULTADOS

3.1. Artigo 1

Prevalence of ColV plasmid-linked genes and in vivo pathogenicity of avian strains of *Escherichia coli*

Artigo submetido à publicação na Revista Foodborne Pathogens and Disease

3.2. Artigo 2

Analysis of prevalence, gene expression and functionality of Type 6 Secretion System in APEC strains

Artigo a ser submetido à publicação

Prevalence of ColV plasmid-linked genes and *in vivo* pathogenicity of avian strains of *Escherichia coli*

Aline Luísa de Oliveira[a,b], Débora Assumpção Rocha[a], Fabrine Finkler[b], Lucas Brunelli de Moraes[b], Nicolle Lima Barbieri[a], Daniel Brisotto Pavanelo[a], Cristina Winkler[a], Tiela Trapp Grassotti[b], Kelly Cristina Tagliari de Brito[b], Benito Guimarães de Brito[b], Fabiana Horn[a*]

[a] Departamento de Biofísica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

[b] Instituto de Pesquisas Veterinárias Desidério Finamor, Eldorado do Sul, RS, Brazil.

*Corresponding author:

Av. Bento Gonçalves, 9500

Porto Alegre/ RS - Brasil

P.O. Box 15005

91501-970

Tel: +55 51 3308 7610

e-mail: fabiana.horn@ufrgs.br

Short title: ColV-linked genes and pathogenicity of avian *E. coli*

ABSTRACT

Avian pathogenic *Escherichia coli* (APEC) cause extraintestinal infections in birds, leading to an increase in the cost of poultry production. The ColV plasmid-linked genes *iroN*, *ompT*, *hlyF*, *iss* and *iutA* have previously been suggested to be predictors of the virulence of APEC. In this research we analyzed the frequencies of these genes in a Brazilian collection of *E. coli* isolated from birds with colibacillosis (APEC) and from apparently healthy birds (Avian fecal (A_{fecal})), as well as from the litter of poultry houses of apparently healthy flocks (Avian litter (A_{litter})). All the isolates that harbored *ompT* also harbored *hlyF*, so they were considered as one trait for statistical analysis. The relationship between *in vivo* virulence in one-day-old chicks, expressed as a pathogenicity score, and the number of genes in each isolate showed that isolates with less than two of the four genes were rarely pathogenic, while many of those with two or more genes were pathogenic. Nevertheless, about half of the isolates harboring two or more genes were non-pathogenic to one-day-old chicks, in agreement with previous observations that commensal *E. coli* isolates from the birds' microbiota can serve as a reservoir of virulence genes. Isolates allocated to phylogenetic group B2, which is frequently associated with extraintestinal infections, had the highest pathogenicity scores, while isolates allocated to group B1 had the lowest.

Keywords: avian pathogenic *Escherichia coli*; APEC; ColV plasmid-linked genes; *in vivo* virulence; pathogenicity; phylogenetic group.

1. INTRODUCTION

The poultry industry generates high-protein food at relatively low cost and is an important branch of the Brazilian economy. The production of chicken meat in Brazil reached 12.3 million tons in 2013 (Ubabef, 2014) with the low cost of production basically due to the relatively low cost of specialized labor, and high grain yields (Alves *et al.*, 2006). However, there are factors that cause a significant decrease in productivity and thus increased costs in the poultry industry; among these are bacterial infections (Fallavena *et al.*, 2000).

Avian pathogenic strains of *Escherichia coli* (APEC) cause extraintestinal infections, collectively known as colibacillosis, which can manifest as lesions in joints, subcutaneous tissue and organs such as air sacs, lungs, liver, and heart. APEC strains are members of the extraintestinal pathogenic *Escherichia coli* (ExPEC) group, which also includes uropathogenic *E. coli* (UPEC) and neonatal meningitis *E. coli* (NMEC) (Russo and Johnson, 2000). Typing of ExPEC strains have shown a close relationship between ExPEC strains of human and avian origin (Ewers et al., 2007; Johnson et al., 2007; Moulin-Schouleur et al., 2007; Rodriguez-Siek et al., 2005a). Birds of 4 to 8 weeks of age are most susceptible to APEC infections. The most evident clinical signs of colibacillosis in birds are depression and fever, which can be followed by death (Nolan *et al.*, 2013). Since the intestinal microbiota of healthy birds can serve as a reservoir of APEC strains, the bacteria may remain in the poultry environment long after an outbreak, leading to contamination of food and water, and serving as a route of pathogen dissemination (Ewers *et al.*, 2009). The intestinal microbiota also harbors *E. coli* strains carrying virulence-associated genes (VAGs), which may be transferable to APEC strains through horizontal gene transfer (Kemmett *et al.*, 2013).

Although the APEC pathotype is not yet completely defined (Dziva e Stevens, 2008), many pathogenicity traits, such as adhesins, serum-resistance factors, iron-acquisition factors and toxins, are known to be associated with these strains (Barbieri *et al.*, 2013). Additionally, large plasmids, such as ColV plasmids, have been associated with *E. coli* virulence (Waters e Crosa, 1991) and are

thought to be important contributors to the APEC pathotype (Ginns *et al.*, 2000; Johnson *et al.*, 2002; Rodriguez-Siek *et al.*, 2005; Johnson *et al.*, 2006). Previous work has shown that five ColV-linked genes, namely *iroN*, *ompT*, *hlyF*, *iss* and *iutA*, occur more frequently in APEC strains than in avian fecal *E. coli* strains, indicating that such genes might be considered predictors of pathogenicity and consequently as identifiers of APEC strains (Johnson *et al.*, 2008).

Since there is no single gene or set of genes exclusively associated with APEC (Dziva e Stevens, 2008), and immunocompromised birds can be infected with less virulent strains and still manifest the disease, *in vivo* assays are valuable in establishing if a strain isolated from a diseased bird is really capable of causing disease in a healthy bird (Schouler *et al.*, 2012; Barbieri *et al.*, 2013).

Here, we analyzed the frequencies of *iroN*, *ompT*, *hlyF*, *iss* and *iutA* in a Brazilian collection of 401 avian *E. coli* isolates (192 APEC (RS and PR), 100 avian litter (A_{litter}) and 109 avian fecal (A_{fecal}). We also determined the phylogenetic group of A_{fecal} and A_{litter} isolates, and the *in vivo* virulence of the A_{litter} isolates. These data were combined with previously established data on the phylogenetic group and *in vivo* virulence of APEC strains (Barbieri *et al.*, 2013; Barbieri *et al.*, 2014). Our aim was to verify whether the presence of the aforementioned genes correlates with the *in vivo* virulence of avian *E. coli* isolates from Brazil.

2. MATERIAL AND METHODS

2.1 Ethics statement

All animal experiments were approved by the Biosafety Committee of the Instituto de Pesquisas Veterinárias Desidério Finamor (CIB 004/08), and chickens were euthanized according to established procedures. The *in vivo* experiments were done in the presence of a qualified veterinarian. This study did not involve endangered or protected species, and no specific permissions were required for collecting avian *E. coli* isolates from broiler chickens.

2.2 Bacterial strains

We evaluated 401 isolates of *E. coli* obtained from different poultry flocks in the Southern Brazilian states of Rio Grande do Sul (RS) and Paraná (PR), which account for 46% of the total Brazilian production (Ubabef, 2014). The APEC strains (*n* = 192) included 140 previously characterized cellulitis isolates (Barbieri *et al.*, 2013) and 52 previously characterized isolates obtained from various organs (skin, air sacs, liver, heart, and intestine) of broiler chickens with signs of colisepticemia (Barbieri *et al.*, 2014).

A_{litter} isolates (*n* = 100) were obtained during the sampling period of November 2011 to April 2012 from 100 distinct flocks of varying ages (4 to 43 days) at various locations in the North and Centre-East regions of RS, Brazil. Isolates (one per flock) were collected by exposing sterile shoe covers to the broiler house floor; the shoe covers were then placed in sterile bags and incubated in BHI at 37 °C for 18-24 h, and a loopful of culture was streaked onto MacConkey agar plates to isolate *E. coli*.

A_{fecal} isolates (*n* = 109) were obtained from January to September 2013 by cloacal swab from apparently healthy 6-to-7-week-old broiler chickens from 7 distinct flocks in the cities of Porto Alegre and Eldorado do Sul, RS.

Biochemical tests (triple sugar iron, urease and sulfide-indole-motility) and characterization in MacConkey agar were performed to confirm that isolates were

E.coli (CLSI, 2009), and they were stored at -80 ^{o}C in Luria-Bertani (LB) broth with 20% glycerol. Isolates are listed in Supplementary Table 1.

2.3 DNA extraction

Strains were streaked on LB (Luria-Bertani) agar and grown at 37 °C overnight. A loop of colonies was suspended in 200 µL of milli-Q water, incubated at -20 °C for 10 min followed by 10 min at 100 °C, and centrifuged at 14,000 *g* for 10 min. The supernatants were used as templates for PCR and stored at 4 °C.

2.4 Genotyping of avian *E. coli* for *iroN, ompT, hlyF, iss,* and *iutA*

E. coli strains were genotyped by multiplex PCR as previously described (Johnson *et al.*, 2008). Reactions were performed in a GenePro Thermal Cycler (Bioer Technology, China) as follows: denaturation for 2 min at 94 °C; 25 cycles of 30 s at 94 °C, 30 s at 63 °C and 3 min at 68 °C, followed by a final extension step of 10 min at 72 °C. PCR products were analyzed by gel electrophoresis, and the sizes of amplicons were determined by comparison with a 100-bp molecular weight marker (Ludwig Biotec, Porto Alegre, Brazil). APEC O1 strain was used as positive control.

2.5 Phylogenetic typing

We performed PCR-based phylogenetic typing of the A_{litter} and A_{fecal} strains as described by Clermont *et. al*. (Clermont *et al.*, 2000) with slight modifications. Reactions were performed in a GenePro Thermal Cycler (Bioer Technology, China) as follows: denaturation for 5 min at 94 °C; 30 cycles of 30 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, followed by a final extension step of 7 min at 72 °C. We used triplex reactions instead of separated reactions for each amplicon. PCR products were analyzed by gel electrophoresis, and the sizes of the amplicons were determined as above.

2.6 Pathogenicity Score of A_{litter} isolates

To assess the ability of the 100 A_{litter} isolates to cause extraintestinal infections, we tested their pathogenicity in one-day-old chicks. A pathogenicity score (PS), which takes into account the time that a chick survives after subcutaneous inoculation (of 10^8 CFU) and the lesions observed at necropsy, was attributed to each strain as described (Barbieri *et al.*, 2012). The PS ranges from 0 (avirulent strain) to 10 (highly virulent strain).

2.7 Statistical analysis

Chi-square test was used to compare the frequencies of the genes among the classes of isolates analyzed (APEC, A_{litter}, A_{fecal}). To do pairwise comparisons of the categories, we used likelihood-ratio tests (adjusted $p \leq 0.005$; Bonferroni correction). Pathogenicity Scores and the number of virulence-associated genes were treated as quantitative variables and described as median ± standard deviation (SD). Data were analyzed using non-parametric tests. The relationship between the presence of genes and PS was analyzed by the Wilcoxon-Mann-Whitney test, and the relationship between PS and phylogenetic group was analyzed using the Kruskal-Wallis test.

3. RESULTS

3.1 Genotyping by pentaplex PCR

We screened 401 avian *E. coli* isolates (APEC, A_{litter} and A_{fecal}) for the presence of *iroN, ompT*, *hlyF*, *iss* and *iutA* genes by pentaplex PCR, as previously described (Johnson *et al.*, 2008), and their frequencies are shown in Figure 1. The frequencies of these genes were significantly lower among the A_{litter} and A_{fecal} isolates in comparison with APEC. All the isolates that harbored *ompT* also harbored *hlyF.* Among the A_{fecal} isolates, no differences could be seen in the frequencies of the genes in isolates obtained from new or reused poultry litter (data not shown), indicating that reuse of the litter did not lead to biased sampling.

When we examined the number of VAGs among the three groups of *E. coli* strains, we observed that the majority (71%) of pathogenic strains harbored the five genes, compared with only 31% of the A_{litter} and 18% of the A_{fecal} strains. These data indicate that these genes occur more frequently in pathogenic strains, but that they can also be present in non-pathogenic strains (Fig. 2B).

3.2 Pathogenicity tests

An avian *E. coli* isolate cannot be considered an APEC on the basis only of the virulence genes it possesses, because no single gene or set of genes has been found to define APEC (Dziva e Stevens, 2008). In order to assess the pathogenicity of A_{litter} isolates, we performed *in vivo* assays, and assigned a PS to each isolate by the method of Souza *et al.* described (Barbieri *et al.*, 2012). The PS values of the A_{litter} *E. coli* isolates ranged from 0 to 5.3: forty-five (45%) isolates had a PS = 0, and only 2 (2%) had a PS > 5.0. The PS for APEC strains had been previously established: of the 192 APEC isolates, 63 (33%) had a median PS = 10; 55 (29%) had a 9.9>median PS>7.0; 48 (25%) had a 6.9>median PS>5.0; and 26 (13%) had a PS lower than 5, among which 4 had a PS = 0 (Barbieri *et al.*, 2013; Barbieri *et al.*, 2014). As expected, the average median PS of A_{litter} isolates (1.08) was significantly lower than the average median PS of APEC isolates (7.48) (previous work) (Barbieri *et al.*, 2013; Barbieri *et al.*, 2014). The PSs of the A_{fecal}

isolates were not determined. When we ordered the strains from highest to lowest PS, we observed a clear separation of APEC and A_{litter} samples, in that APEC isolates had the highest PS values, while the A_{litter} isolates had the lowest, with a few exceptions (Fig. 2B).

3.3 Phylogenetic typing

We performed phylogenetic typing of the 100 A_{litter} and 109 A_{fecal} *E. coli* isolates based on the method described by Clermont *et. al*. (Clermont *et al.*, 2000), which assigns *E. coli* strains to four main phylogenetic groups A, B1, B2 and D, according to the presence of the genes *chuA* and *yjaA* and the DNA fragment *TSPE4.C2*. The A_{litter} isolates were mainly allocated to phylogenetic groups B1 (45%) and A (41%), with the remaining isolates classified as group D (12%) and B2 (2%). The A_{fecal} isolates mainly belonged to phylogenetic groups D (50%) and A (29%), with the remaining isolates assigned to groups B1 (17%) and B2 (4%). The APEC strains had been previously allocated to group D (44%) and A (29%), followed by groups B2 (17 %) and B1 (10%) (Barbieri *et al.*, 2013; Barbieri *et al.*, 2014). These results are summarized in Table 1. The distribution of APEC, A_{litter} and A_{fecal} isolates by phylogenetic group is shown in Figure 2A. When the APEC and A_{litter} isolates are ordered according to their PS, it can be seen that strains belonging to phylogenetic groups B2 and D are concentrated in the upper region of the figure, which comprises isolates with higher PS values (Fig. 2B).

3.4 Statistical analysis

The chi-square test showed that the frequencies of the genes differed statistically among APEC, A_{litter} and A_{fecal} isolates, and the likelihood-ratio tests showed that all genes were significantly more frequent in APEC than in A_{litter} and A_{fecal} isolates (adjusted $p \leq 0.005$; Bonferroni correction) (Fig. 1).

We used the Wilcoxon-Mann-Whitney test to determine for each of the five genes if its presence in the combined APEC and A_{litter} isolates was related to PS. This was indeed the case (Table 2). Since all isolates that harbored *ompT* also harbored *hlyF*, we considered them one gene, and performed subsequent

analyses taking four genes (rather than five) as the maximum number of genes per isolate.

When we assigned the APEC and A_{litter} samples to one of two groups, those with 0 or 1 genes, and those with 2-4 genes, the difference between the two groups in terms of PS was highly significant ($p \leq 0.001$; Kruskall-Wallis test) (Table 2). In other words, avirulent isolates (median PS ≤ 1.1) tend to harbor 0 or 1 gene, while virulent isolates (median PS ≥ 6.3) tend to harbor 2, 3 or 4 genes. When we used this criterion to classify our isolates, we found that 95% of the APEC isolates indeed fell into group 2 (Table 3). On the other hand, 51% of the A_{litter} isolates (average median PS = 1.08) also possessed 2 - 4 genes, thus falling into the same group as the pathogenic isolates (Table 3). Because the high proportion of A_{litter} isolates harboring 2 to 4 genes might reflect some bias in strain collection, we assembled additional 109 A_{fecal} isolates from the cloacae of healthy broiler chickens. Again, 58 (53%) of the 109 of these isolates possessed 2-4 genes.

We also used the Kruskall-Wallis test to analyze the relationship between PS and the phylogenetic groups of the isolates. This showed that isolates belonging to groups B2 and D had higher median PS (9.7 and 6.8, respectively), while those belonging to groups A and B1 had lower median PS (Table 4).

4. DISCUSSION

In the present work, we tested whether the pentaplex PCR for the Col V plasmid-linked genes *iroN*, *ompT*, *hlyF*, *iss* and *iutA* could be used as a rapid diagnostic tool to discriminate APEC from A_{litter} and A_{fecal} isolates of a Brazilian collection. The frequencies of these genes were significantly higher among APEC (82-95%) in comparison with A_{litter} (40-53%) and A_{fecal} (20-76%) isolates (Fig. 1). Among our APEC strains, the frequencies were similar to the frequencies among APEC strains from the USA (78-85%) (Johnson et al., 2008), Egypt (90-94%) (Hussein et al., 2013) and Brazil (68-72%) (Kobayashi et al., 2011). Likewise, the frequencies of these genes among our A_{litter} and A_{fecal} strains were similar to those found among A_{fecal} strains from Egypt (47-53%) (Hussein et al., 2013) and among *E. coli* isolated from retailed chicken from Brazil (27-68%) (Kobayashi et al., 2011); however, they were higher than those found among A_{fecal} from the USA (21-35%) (Johnson et al., 2008).

Large conjugative ColV plasmids carry well-known virulence markers of APEC (Ginns et al., 2000), and they have been observed to be more frequent in APEC than in A_{fecal} strains (Johnson et al., 2006). Indeed, 71% of our APEC isolates harbored the five pentaplex genes, while only 31% of A_{litter} and 25% of A_{fecal} isolates harbored the five genes.

In vivo assays were performed to analyze the virulence of the A_{litter} isolates. As expected, the average median PS of APEC isolates (Barbieri et al., 2013; Barbieri et al., 2015) was significantly higher (7.48) than the average median PS of A_{litter} isolates (1.08). To test whether the presence of pentaplex genes may influence the PS, we correlated the presence of these genes with the PS of the samples. In agreement with Johnson et al (Johnson et al., 2008), we found that the presence of each gene was associated with a higher median PS, while its absence was associated with a lower median PS (Table 1). We also found that avirulent isolates (PS ≤1.1) harbored 0 or only 1 gene, while virulent isolates (PS ≥ 6.3) harbored 2, 3 or 4 genes (Table 2). When we applied this classification to our isolates, we found that 95% of the APEC isolates were correctly classified into

group 2 (2, 3 or 4 genes) (Table 3). Interestingly, among the nine cellulitis and colisepticemic isolates that harbored 0 or 1 gene (group 1), three (RS025, RS032 and RS107) had a PS = 1, and one (RS111) had a PS = 3.1, indicating that despite having been isolated from diseased birds, these strains were not able to cause disease in healthy birds. The remaining five isolates (PR010, PR033, PR117, RS023 and RS045) had a PS > 6.3 (Table S1).

However, only 49% of the non-pathogenic A_{litter} isolates (average median PS = 1.08) were classified into group 1 (none or 1 gene, Table 3). That is, 51% of the non-pathogenic A_{litter} isolates possessed 2, 3 or 4 genes, and would incorrectly be classified as APEC if the pentaplex criterion was applied. A similar picture was observed among the A_{fecal} isolates, since 53% possessed 2, 3 or 4 genes and would also be incorrectly classified as APEC. Although the virulence of A_{fecal} isolates were not assessed *in vivo* and the gastrointestinal microbiota can serve as a reservoir of APEC (Ewers 2009), it is highly unlikely that 53% of *E. coli* isolated from the intestine of healthy broilers would be APEC. This relatively high frequency of *iroN*, *ompT*, *hlyF*, *iss* and *iutA* genes among the A_{fecal} and A_{litter} isolates is surprising, but is consistent with the fact that gastrointestinal microbiota can serve as a reservoir of APEC VAGs (Kemmett et al., 2013). This also suggests that commensal isolates from the microbiota of birds can serve as a reservoir of transferable virulence genes that can eventually emerge among intestinal APEC to facilitate the emergence of pathogenic strains by horizontal gene transfer.

We have also performed the Clermont phylogenetic typing to access the distribution of our isolates among the four main phylogenetic groups. Isolates with higher PS belong mainly to groups B2 and D, while isolates with a lower PS belong mainly to groups A and B1 (Fig. 2B); moreover, A_{litter} strains belonged mainly to groups A (41%) and B1 (45%). These results agree with previous works showing that extraintestinal virulent strains tend to belong to groups B2 and D, while commensal strains tend to fall to groups A and B1 (Bingen et al., 1998; Johnson et al., 2001; Picard et al., 1999).

5. CONCLUSION

The presence of the ColV-linked genes*, iroN*, *ompT*, *hlyF*, *iss* and *iutA*, proposed as minimal predictors of APEC virulence, in Brazilian APEC and avian litter (A_{litter}) *E. coli* isolates, is associated with *in vivo* pathogenicity of the isolates. Pathogenic strains generally harbor two or more of these genes while isolates with less than two genes are rarely pathogenic. However, approximately 50% of the non-pathogenic isolates also harbor two or more of the genes; thus the presence of any two or more genes is necessary but not sufficient to turn an *E. coli* into an APEC.

ACKNOWLEDGEMENTS

We warmly thank Lisa K. Nolan and Jorge A. Girón for critically reading the manuscript and for their useful suggestions. This work was supported by the CNPq (MAPA 578364/2008-5 and MAPA 578440/2008-3); A.L.O. has been supported by a CNPq Master Degree studentship.

REFERENCES

Alves, J.M.d.S., Martinelli, O., Dewes, H., 2006. Dinâmica inovativa no agronegócio: a inovação tecnológica na avicultura industrial por meio da análise de patentes. Cadernos de Ciência & Tecnologia 23, 207-233.

Barbieri, N.L., de Oliveira, A.L., Tejkowski, T.M., Pavanelo, D.B., Rocha, D.A., Matter, L.B., Callegari-Jacques, S.M., de Brito, B.G., Horn, F., 2013. Genotypes and pathogenicity of cellulitis isolates reveal traits that modulate APEC virulence. PLoS One 8, e72322.

Barbieri, N.L., Oliveira, A.L., Tejkowski, T.M., Pavanelo, D.B., Matter, L.B., Pinheiro, S.R.S., Vaz, T.M.I., Brito, B.G., Nolan, L.K., Logue, C.M., Horn, F., 2015. Molecular characterization and clonal relationships among *Escherichia coli* strains isolated from broiler chickens with colisepticemia. Foodborne Pathogens and Disease 12, 74-83.

Barbieri, N.L., Tejkowski, T.M., Oliveira, A.L., Brito, B.G., Horn, F., 2012. Characterization of extra-intestinal *Escherichia coli* isolated from a peacock (*Pavo cristatus*) with colisepticemia. Avian Diseases *56*, 436-440.

Bingen, E., Picard, B., Brahimi, N., Mathy, S., Desjardins, P., Elion, J., Denamur, E., 1998. Phylogenetic analysis of *Escherichia coli* strains causing neonatal meningitis suggests horizontal gene transfer from a predominant pool of highly virulent B2 group strains. The Journal of Infectious Diseases 177, 642-650.

Clermont, O., Bonacorsi, S., Bingen, E., 2000. Rapid and simple determination of the *Escherichia coli* phylogenetic group. Applied and Environmental Microbiology 66, 4555-4558.

CLSI 2009. Performance Standards for Antimicrobial Susceptibility Testing; Aproved Standard- Tenth Edition M02-A10 (940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA, Clinical and Laboratory Standards Institute).

Dziva, F., Stevens, M.P., 2008. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathology 37, 355-366.

Ewers, C., Antao, E.-M., Diehl, I., Philipp, H.-C., Wieler, L.H., 2009. Intestine and environment of the chicken as reservoirs for extraintestinal pathogenic *Escherichia coli* strains with zoonotic potential. Applied and Environmental Microbiology 75, 184-192.

Ewers, C., Li, G.W., Wilking, H., Kiessling, S., Alt, K., Antao, E.M., Laturnus, C., Diehl, I., Glodde, S., Homeier, T., Bohnke, U., Steinruck, H., Philipp, H.C., Wieler, L.H., 2007. Avian pathogenic, uropathogenic, and newborn meningitis-causing *Escherichia coli*: How closely related are they? International Journal of Medical Microbiology 297, 163-176.

Fallavena, L.C.B., Moraes, H.L.S., Salle, C.T.P., da Silva, A.B., Vargas, R.S., do Nascimento, V.P., Canal, C.W., 2000. Diagnosis of skin lesions in condemned or downgraded broiler carcasses - a microscopic and macroscopic study. Avian Pathology 29, 557-562.

Ginns, C.A., Benham, M.L., Adams, L.M., Whithear, K.G., Bettelheim, K.A., Crabb, B.S., Browning, G.F., 2000. Colonization of the respiratory tract by a virulent strain of avian *Escherichia coli* requires carriage of a conjugative plasmid. Infection and Immunity 68, 1535-1541.

Hussein, A.H., Ghanem, I.A., Eid, A.A., Ali, M.A., Sherwood, J.S., Li, G., Nolan, L.K., Logue, C.M., 2013. Molecular and phenotypic characterization of *Escherichia coli* isolated from broiler chicken flocks in Egypt. Avian Dis 57, 602-611.

Johnson, J.R., Delavari, P., Kuskowski, M., Stell, A.L., 2001. Phylogenetic distribution of extraintestinal virulence-associated traits in *Escherichia coli*. J Infect Dis 183, 78-88.

Johnson, T.J., Giddings, C.W., Horne, S.M., Gibbs, P.S., Wooley, R.E., Skyberg, J., Olah, P., Kercher, R., Sherwood, J.S., Foley, S.L., Nolan, L.K., 2002. Location of increased serum survival gene and selected virulence traits on a conjugative R plasmid in an avian *Escherichia coli* isolate. Avian Diseases 46, 342-352.

Johnson, T.J., Kariyawasam, S., Wannemuehler, Y., Mangiamele, P., Johnson, S.J., Doetkott, C., Skyberg, J.A., Lynne, A.M., Johnson, J.R., Nolan, L.K., 2007. The genome sequence of avian pathogenic *Escherichia coli* strain O1 : K1 : H7 shares strong similarities with human extraintestinal pathogenic *E. coli* genomes. Journal of Bacteriology 189, 3228-3236.

Johnson, T.J., Siek, K.E., Johnson, S.J., Nolan, L.K., 2006. DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. Journal of Bacteriology 188, 745-758.

Johnson, T.J., Wannemuehler, Y., Doetkott, C., Johnson, S.J., Rosenberger, S.C., Nolan, L.K., 2008. Identification of minimal predictors of avian pathogenic *Escherichia coli*

virulence for use as a rapid diagnostic tool. Journal of Clinical Microbiology 46, 3987-3996.

Kemmett, K., Humphrey, T., Rushton, S., Close, A., Wigley, P., Williams, N.J., 2013. A longitudinal study simultaneously exploring the carriage of APEC virulence associated genes and the molecular epidemiology of faecal and systemic *E. coli* in commercial broiler chickens. PLoS One 8.

Kobayashi, R.K.T., Aquino, I., Ferreira, A.L.d.S., Vidotto, M.C., 2011. EcoR phylogenetic analysis and virulence genotyping of Avian Pathogenic *Escherichia coli* strains and *Escherichia coli* isolates from commercial chicken carcasses in Southern Brazil. Foodborne Pathogens and Disease 8, 631-634.

Moulin-Schouleur, M., Reperant, M., Laurent, S., Bree, A., Mignon-Grasteau, S., Germon, P., Rasschaert, D., Schouler, C., 2007. Extraintestinal pathogenic *Escherichia coli* strains of avian and human origin: Link between phylogenetic relationships and common virulence patterns. Journal of Clinical Microbiology 45, 3366-3376.

Nolan, L.K., Barnes, H.J., Vaillancourt, J.P., Abdul-Aziz, T., Logue, C.M. 2013. Colibacillosis, In: Swayne, D.E., Glisson, J.R., McDougald, L.R., Nolan, L.K., Suarez, D.L., Nair, V.L. (Eds.) Diseases of Poultry. Wiley-Blackwell, Ames, Iowa, USA.

Picard, B., Garcia, J.S., Gouriou, S., Duriez, P., Brahimi, N., Bingen, E., Elion, J., Denamur, E., 1999. The link between phylogeny and virulence in *Escherichia coli* extraintestinal infection. Infection and Immunity 67, 546-553.

Rodriguez-Siek, K.E., Giddings, C.W., Doetkott, C., Johnson, T.J., Fakhr, M.K., Nolan, L.K., 2005a. Comparison of *Escherichia coli* isolates implicated in human urinary tract infection and avian colibacillosis. Microbiology-Sgm 151, 2097-2110.

Rodriguez-Siek, K.E., Giddings, C.W., Doetkott, C., Johnson, T.J., Nolan, L.K., 2005b. Characterizing the APEC pathotype. Veterinary Research 36, 241-256.

Russo, T.A., Johnson, J.R., 2000. Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. Journal of Infectious Diseases 181, 1753-1754.

Schouler, C., Schaeffer, B., Brée, A., Mora, A., Dahbi, G., Biet, F., Oswald, E., Mainil, J., Blanco, J., Moulin-Schouleur, M., 2012. Diagnostic strategy for identifying avian pathogenic *Escherichia coli* based on four patterns of virulence genes. Journal of Clinical Microbiology 50, 1673-1678.

UBABEF 2014. União Brasileira de Avicultura, Relatório Anual 2014 (www.ubabef.com.br).

Waters, V.L., Crosa, J.H., 1991. Colicin V virulence plasmids. Microbiological reviews 55, 437-450.

LEGENDS TO FIGURES

Figure 1. Histograms comparing the frequencies of genes *iroN, ompT/hlyF, iss, iutA* in APEC, A_{litter} and A_{fecal} isolates. Letters above bars indicate levels of statistical significance according to likelihood-ratio tests (adjusted $p \leq 0.005$; Bonferroni correction).

Figure 2. (**A**) APEC, A_{litter} and A_{fecal} isolates ordered according to number of genes harbored (from higher to lower). The column on the left gives the origins of the isolates: APEC (red), A_{litter} (dark green) and A_{fecal} (light green). (**B**) APEC and A_{litter} isolates ordered according to their PS (higher to lower). The column on the left gives the origins of the isolates: APEC (red) and A_{litter} (green). Columns 2 to 6 show the genotype of each isolate with respect to the pentaplex genes (black, presence of the respective gene; white, absence). The column on the right indicates the phylogenetic group of each isolate: group A, dark blue; group B1, light blue; group B2, dark red; group D, light orange. The phylogenetic grouping and PS of the APEC strains was obtained from previous publications (Barbieri *et al.*, 2013; Barbieri *et al.*, 2014).

FIGURES

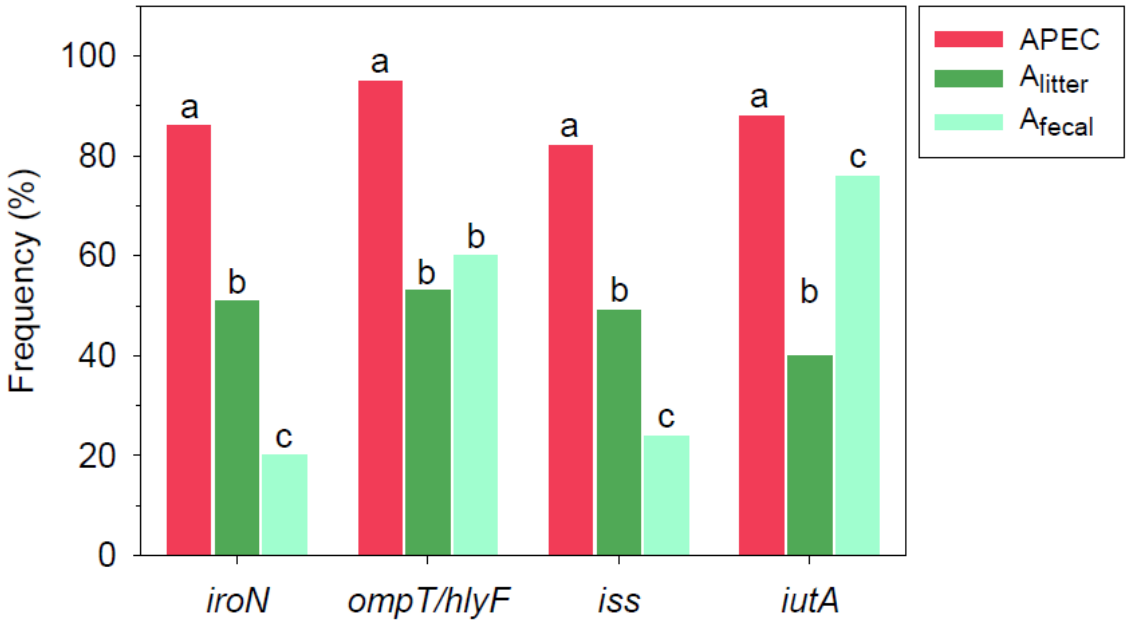


Figure 1. Histograms comparing the frequencies of genes *iroN, ompT/hlyF, iss, iutA* in APEC, A_{litter} and A_{fecal} isolates. Letters above bars indicate levels of statistical significance according to likelihood-ratio tests (adjusted $p \leq 0.005$; Bonferroni correction).

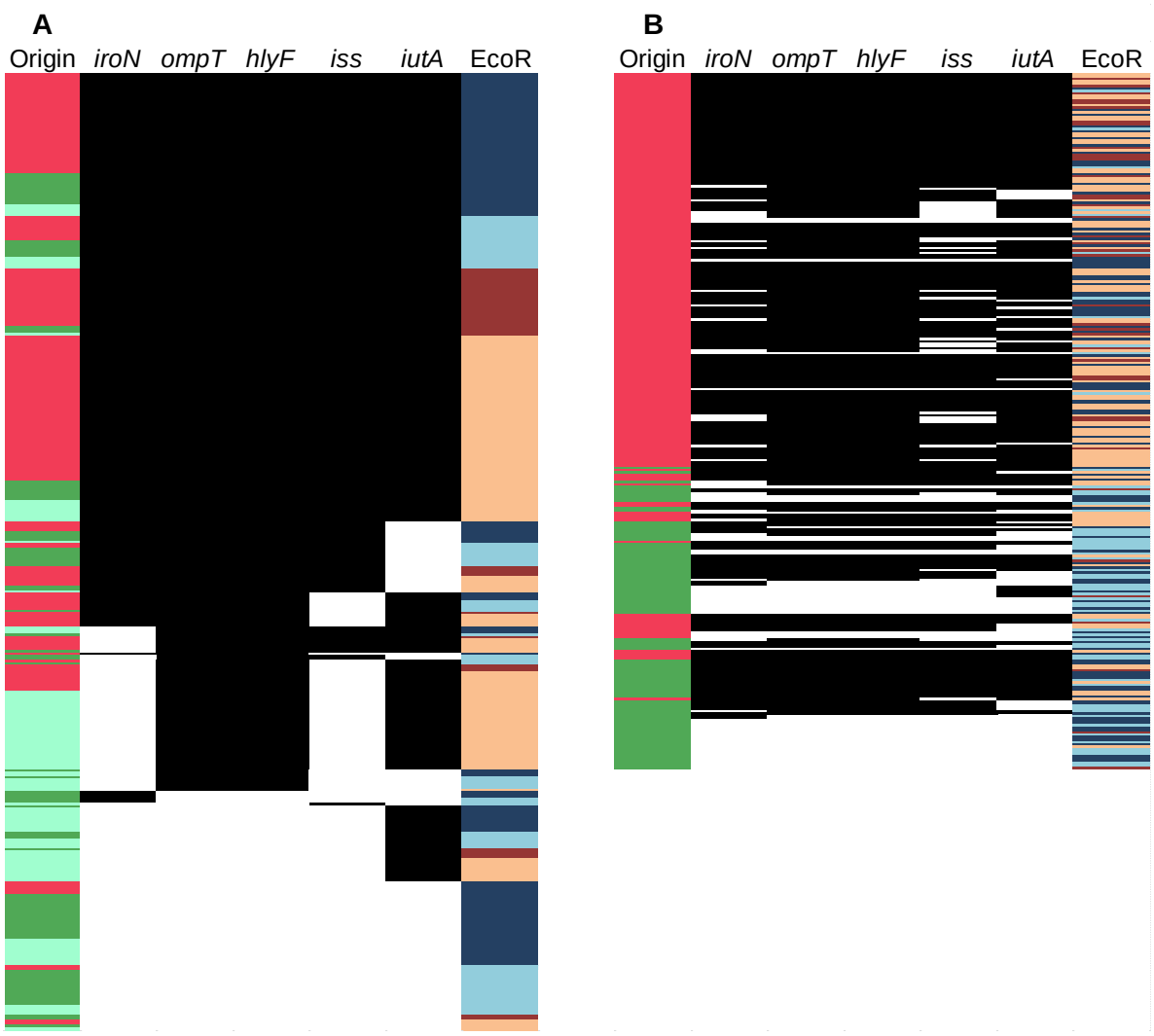


Figure 2. (**A**) APEC, A_{litter} and A_{fecal} isolates ordered according to number of genes harbored (from higher to lower). The column on the left gives the origins of the isolates: APEC (red), A_{litter} (dark green) and A_{fecal} (light green). (**B**) APEC and A_{litter} isolates ordered according to their PS (higher to lower). The column on the left gives the origins of the isolates: APEC (red) and A_{litter} (green). Columns 2 to 6 show the genotype of each isolate with respect to the pentaplex genes (black, presence of the respective gene; white, absence). The column on the right indicates the phylogenetic group of each isolate: group A, dark blue; group B1, light blue; group B2, dark red; group D, light orange. The phylogenetic grouping and PS of the APEC strains was obtained from previous publications (Barbieri *et al.*, 2013; Barbieri *et al.*, 2014).

Table 1. Allocation of APEC, A_{litter} and A_{fecal} isolates in phylogenetic groups A, B1, B2 and D.

Phylogenetic Group	APEC (n=192)	A_{litter} (n=100)	A_{fecal} (n=109)
A	29%	41%	29%
B1	10%	45%	17%
B2	17%	2%	4%
D	44%	12%	50%

Table 2. Relationship between Pathogenicity Score (PS) and the presence of different genes in combined APEC and Alitter isolates.

		Gene +		Gene -		
Gene	Description	Median PS[a]	Number of isolates	Median PS[a]	Number of isolates	p[b]
iroN	Salmochelin receptor	6.93	216	1.1	76	5.6×10^{-8} *
ompT	Episomal outer membrane protease	6.93	236	1.1	56	3.8×10^{-15} *
hlyF	Putative hemolysin	6.93	236	1.1	56	3.8×10^{-15} *
iss	Episomal increased serum survival	6.79	206	1.1	86	2.1×10^{-6} *
iutA	Aerobactin receptor	7.0	208	1.1	84	5.9×10^{-14} *

[a] The PS ranges from 0 (avirulent strain) to 10 (highly virulent strain)
[b] Exact *p* values for Wilcox-Mann-Whitney Test

Table 3. Allocation of the APEC and Alitter isolates to two groups according to Pathogenicity Score (PS) and number of genes harbored.

Group	Number of genes	Number of isolates	Median PS
1	0	46	1.0
	1	12	1.1
2	2	16	7.3
	3	50	6.3
	4	168	6.9

Kruskall-Wallis test; $p \leq 0.001$

Table 4. Correlation between groups 1 (with 0 or 1 gene) and 2 (with 2, 3 or 4 genes) and the origin of strains

Origin	Group 1	Group 2	Total
Cellulitis or colisepticemic *E. coli* or APEC (192)	9	183	192
Non-pathogenic A_{litter} isolates (100)	49	51	100

Sensitivity: 0.95

Specificity: 0.49

Supplementary Table 1.Characterization of 401 avian *E. coli* isolates in terms of the presence of *iroN, ompT, hlyF, iss* and *iutA* genes, phylogenetic group and PS.

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS[1]
PR001	1	1	1	1	1	D	6,07
PR002	0	1	1	0	1	D	10,00
PR003	1	1	1	0	1	A	6,29
PR004	1	1	1	1	1	D	10,00
PR006	1	1	1	1	1	A	7,29
PR007	1	1	1	1	0	B1	7,57
PR008	0	1	1	1	1	D	2,50
PR009	0	1	1	1	1	D	6,29
PR010	0	0	0	0	0	A	9,15
PR011	1	1	1	1	1	D	10,00
PR012	1	1	1	1	1	A	6,93
PR013	1	1	1	1	1	B2	10,00
PR014	1	1	1	1	1	B1	3,85
PR015	0	1	1	0	1	D	5,57
PR016	0	1	1	0	1	D	7,57
PR017	1	1	1	1	1	B2	9,29
PR018	1	1	1	0	1	A	7,29
PR019	1	1	1	0	1	B1	7,93
PR020	1	1	1	1	0	A	7,79
PR021	1	1	1	1	1	A	9,29
PR22	1	1	1	1	1	D	4,71
PR23	1	1	1	1	1	D	8,57
PR24	1	1	1	1	1	D	2,50
PR25	1	1	1	1	1	D	8,65
PR26	1	1	1	1	1	B2	5,78
PR27	1	1	1	1	1	D	10,00
PR28	1	1	1	1	1	D	10,00
PR29	1	1	1	1	1	B2	10,00
PR30	0	1	1	0	1	B1	10,00
PR031	1	1	1	1	1	A	10,00
PR032	1	1	1	1	1	D	6,93
PR033	0	0	0	0	0	B1	7,07
PR034	1	1	1	1	1	B1	10,00
PR035	1	1	1	1	1	B2	10,00
PR036	1	1	1	1	1	D	10,00
PR038	1	1	1	1	1	D	6,29
PR039	1	1	1	1	1	D	10,00
PR040	1	1	1	1	1	B2	10,00

Strain	*ironN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS[1]
PR041	0	1	1	1	1	D	10,00
PR042	1	1	1	1	1	B2	7,29
PR043	1	1	1	1	1	B2	10.00
PR044	1	1	1	1	1	D	10.00
PR045	1	1	1	1	1	D	8.65
PR046	1	1	1	1	1	B2	10.00
PR047	1	1	1	1	1	D	9.65
PR048	1	1	1	1	1	B1	5.15
PR049	1	1	1	1	1	D	7.29
PR050	1	1	1	1	1	A	10.00
PR051	1	1	1	1	0	D	7.29
PR052	1	1	1	0	1	D	6.29
PR053	1	1	1	0	1	D	10.00
PR054	1	1	1	1	1	D	6.43
PR055	1	1	1	1	1	D	10.00
PR056	0	1	1	0	1	B2	6.29
PR057	0	1	1	0	1	B2	10.00
PR058	1	1	1	1	1	D	5.57
PR059	1	1	1	1	0	D	10.00
PR061	1	1	1	1	1	A	10.00
PR062	1	1	1	1	1	D	10.00
PR064	1	1	1	1	1	D	9.65
PR065	1	1	1	1	0	A	10.00
PR068	1	1	1	1	1	D	10.00
PR069	1	1	1	1	1	A	6.29
PR070	1	1	1	1	1	B2	10.00
PR071	1	1	1	1	1	B2	9.29
PR072	1	1	1	1	1	B2	10.00
PR073	1	1	1	0	0	A	9.65
PR074	1	1	1	1	1	A	8.07
PR075	1	1	1	1	0	B2	10.00
PR076	1	1	1	0	1	D	7.29
PR077	1	1	1	1	1	A	6.29
PR078	1	1	1	1	1	A	10.00
PR079	1	1	1	1	1	B1	10.00
PR080	1	1	1	1	1	D	8.29
PR081	1	1	1	1	1	A	10.00
PR082	1	1	1	1	1	A	9.65
PR083	1	1	1	1	1	D	10.00
PR084	1	1	1	1	1	D	5.93
PR085	1	1	1	1	1	D	10.00

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS[1]
PR086	1	1	1	1	1	A	8.21
PR087	1	1	1	1	1	D	5.35
PR088	1	1	1	1	1	A	10.00
PR089	1	1	1	1	0	A	5.93
PR091	1	1	1	1	1	A	7.57
PR092	0	1	1	1	1	B2	7.85
PR093	1	1	1	1	1	D	9.65
PR094	1	1	1	1	1	D	8.65
PR095	1	1	1	1	1	D	10.00
PR096	1	1	1	1	1	D	6.29
PR097	1	1	1	1	1	D	6.22
PR098	1	1	1	1	1	B1	6.36
PR099	1	1	1	1	1	D	10.00
PR100	1	1	1	1	1	A	5.93
PR101	1	1	1	1	1	D	6.36
PR102	1	1	1	1	1	A	10.00
PR103	1	1	1	1	1	A	6.43
PR104	1	1	1	1	1	B2	10.00
PR105	1	1	1	1	1	A	6.22
PR106	1	1	1	1	1	D	10.00
PR107	1	1	1	1	1	D	6.29
PR108	1	1	1	1	1	A	10.00
PR109	1	1	1	1	1	D	5.93
PR110	1	1	1	1	1	B2	6.93
PR111	1	1	1	1	0	B2	6.57
PR112	1	1	1	1	1	A	4.93
PR113	1	1	1	1	1	D	6.07
PR114	1	1	1	1	1	A	9.65
PR115	1	1	1	1	1	B2	10.00
PR116	1	1	1	1	1	D	8.29
PR117	0	0	0	0	0	A	10.00
PR118	1	1	1	1	1	A	4.71
PR119	1	1	1	1	1	D	6.79
PR120	1	1	1	1	1	B1	2.00
PR121	1	1	1	1	1	D	5.57
PR122	1	1	1	1	1	D	2.50
PR123	1	1	1	1	1	B2	10.00
PR124	1	1	1	1	1	D	5.57
PR125	0	1	1	0	1	B2	6.29
PR126	1	1	1	1	1	D	5.71
PR127	1	1	1	1	1	D	6.93

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS[1]
PR128	1	1	1	1	1	B2	10.00
PR129	1	1	1	1	1	D	1.00
PR130	1	1	1	1	1	A	6.43
PR131	1	1	1	1	1	D	3.85
PR132	1	1	1	1	1	A	10.00
PR133	1	1	1	1	1	D	7.43
PR134	1	1	1	1	1	D	5.93
PR135	1	1	1	1	0	B2	10.00
PR136	1	1	1	1	1	A	8.79
PR137	1	1	1	1	1	D	6.79
PR138	1	1	1	1	0	B2	7.43
PR139	1	1	1	1	1	A	10.00
PR140	1	1	1	1	1	A	6.93
PR142	1	1	1	1	1	B1	10.00
PR143	1	1	1	1	1	D	10.00
PR144	1	1	1	1	1	D	6.93
PR145	1	1	1	1	1	D	10.00
PR146	1	1	1	1	1	A	8.65
PR147	1	1	1	1	1	D	6.79
PR148	1	1	1	1	1	A	10.00
RS001	1	1	1	1	0	A	7.93
RS002	1	1	1	1	0	D	1.00
RS003	0	1	1	1	1	D	10.00
RS004	1	1	1	1	1	B2	7.07
RS005	1	1	1	1	1	B2	9.15
RS007	1	1	1	0	1	A	10.00
RS008	1	1	1	0	1	B2	10.00
RS009	1	1	1	0	1	D	10.00
RS010	1	1	1	0	1	B1	9.29
RS011	1	1	1	0	1	B1	10.00
RS012	1	1	1	0	1	B1	7.29
RS013	1	1	1	1	1	D	1.00
RS017	0	1	1	0	1	D	9.65
RS018	1	1	1	1	1	A	0.00
RS020	0	1	1	0	1	D	5.93
RS021	1	1	1	1	1	B1	1.00
RS022	1	1	1	1	0	D	1.00
RS023	0	0	0	0	0	A	6.43
RS024	1	1	1	1	1	B2	1.00
RS025	0	0	0	0	0	A	1.00
RS026	1	1	1	1	1	B1	0.00

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS[1]
RS027	1	1	1	1	1	B1	0.00
RS028	1	1	1	1	1	B2	10.00
RS029	1	1	1	1	1	B2	7.43
RS030	1	1	1	1	1	A	6.29
RS031	1	1	1	1	1	D	10.00
RS032	0	0	0	0	0	B1	1.00
RS033	1	1	1	1	1	A	8.79
RS037	1	1	1	1	1	D	10.00
RS038	1	1	1	1	1	A	7.85
RS039	1	1	1	1	0	B1	1.00
RS040	1	1	1	1	1	A	8.57
RS042	1	1	1	1	1	B2	6.71
RS043	1	1	1	1	1	D	0.50
RS044	1	1	1	1	1	A	7.57
RS045	0	0	0	0	0	D	10.00
RS046	1	1	1	0	1	D	0.00
RS047	0	1	1	1	1	D	4.50
RS048	1	1	1	1	1	B2	9.65
RS049	1	1	1	1	1	A	8.93
RS050	1	1	1	1	1	A	7.71
RS071	0	1	1	0	1	D	7.07
RS084	1	1	1	1	1	D	6.42
RS085	1	1	1	1	1	A	8.65
RS086	1	1	1	1	1	A	10.00
RS087	1	1	1	1	1	A	9.15
RS096	1	1	1	1	1	D	5.43
RS097	1	1	1	1	1	A	7.43
RS106	0	1	1	0	1	D	8.29
RS107	0	0	0	0	0	A	1.00
RS108	0	1	1	0	1	D	9.29
RS111	0	0	0	0	0	D	3.14
ECO1	1	0	0	0	0	B1	0.00
ECO2	0	0	0	0	0	A	0.00
ECO4	0	0	0	0	0	A	0.00
ECO5	0	1	1	1	1	D	4.65
ECO6	0	0	0	0	0	B1	0.00
ECO7	1	1	1	1	0	A	0.00
ECO8	1	1	1	1	0	A	0.00
ECO9	1	1	1	1	1	B2	4.30
ECO10	1	1	1	1	1	A	0.00
ECO11	1	1	1	1	1	A	0.00

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS[1]
ECO12	0	0	0	0	0	A	0.00
ECO13	0	0	0	0	0	A	0.00
ECO14	0	0	0	0	0	A	2.10
ECO15	1	1	1	1	1	D	1.10
ECO16	1	1	1	1	1	D	0.00
ECO17	0	0	0	0	0	A	4.30
ECO18	0	0	0	0	0	B1	4.45
ECO19	0	0	0	0	0	B1	0.00
ECO20	0	0	0	0	0	A	4.30
ECO21	1	1	1	1	1	D	0.00
ECO22	0	0	0	0	0	B1	0.00
ECO23	0	0	0	0	0	A	0.00
ECO24	0	0	0	0	0	A	0.00
ECO25	0	0	0	0	0	A	4.10
ECO26	0	0	0	0	0	B1	0.00
ECO27	1	1	1	1	1	B1	0.00
ECO28	0	0	0	0	0	A	0.00
ECO29	0	0	0	0	0	B1	1.15
ECO31	1	1	1	1	1	B1	4.30
ECO34	1	0	0	0	0	A	0.00
ECO36	1	1	1	1	0	B1	0.00
ECO37	0	0	0	0	0	D	0.00
ECO40	1	1	1	1	1	A	0.00
ECO42	1	1	1	1	0	B1	0.00
ECO43	1	1	1	1	1	A	0.00
ECO44	1	1	1	1	1	A	0.00
ECO45	1	1	1	1	1	B1	1.60
ECO46	0	0	0	0	0	B1	0.00
ECO47	1	1	1	1	1	B1	0.00
ECO48	1	1	1	1	1	D	0.00
ECO50	0	0	0	0	0	B1	0.00
ECO51	0	0	0	0	0	B1	0.00
ECO53	1	1	1	1	1	B1	0.00
ECO55	1	1	1	0	1	B1	1.10
ECO57	1	1	1	1	0	D	2.35
ECO61	1	1	1	1	0	B1	0.60
ECO62	1	1	1	1	1	A	0.00
ECO63	1	1	1	1	1	A	0.00
ECO64	1	1	1	1	1	D	0.00
ECO66	1	1	1	1	1	D	0.00
ECO67	0	0	0	0	0	A	0.00

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS
ECO68	1	1	1	1	1	A	0.00
ECO70	0	1	1	1	1	B1	0.00
ECO71	1	1	1	1	1	A	0.60
ECO73	1	1	1	1	0	A	1.10
ECO75	1	1	1	1	0	A	0.00
ECO76	1	1	1	1	1	B1	1.10
ECO77	1	1	1	1	1	B2	1.10
ECO80	0	0	0	0	0	A	1.60
ECO81	0	1	1	0	1	B1	4.30
ECO83	0	0	0	0	0	A	0.00
ECO84	0	0	0	0	0	A	0.00
ECO85	0	0	0	0	0	B1	1.10
ECO87	0	1	1	0	0	A	1.10
ECO90	1	0	0	0	0	A	1.10
ECO91	1	1	1	1	1	B1	0.60
ECO93	1	1	1	1	1	B1	2.10
ECO94	1	0	0	0	0	B1	1.10
ECO95	0	0	0	0	1	B1	1.10
ECO97	0	0	0	0	0	A	1.10
ECO99	1	1	1	1	1	A	3.65
ECO102	1	1	1	1	0	B1	1.10
ECO103	0	0	0	0	0	B1	0.00
ECO104	1	1	1	1	0	B1	2.10
ECO106	1	1	1	1	1	A	1.10
ECO107	0	0	0	0	1	B1	1.10
ECO108	1	0	0	0	0	A	2.15
ECO109	0	0	0	0	1	A	1.10
ECO110	1	1	1	1	1	D	1.10
ECO111	0	0	0	0	1	B1	1.10
ECO113	1	1	1	1	1	D	2.15
ECO116	1	1	1	1	1	A	5.30
ECO118	1	1	1	1	1	A	1.10
ECO119	0	0	0	0	0	A	0.60
ECO122	0	0	0	0	0	B1	1.10
ECO123	1	1	1	1	0	D	5.05
ECO124	0	0	0	0	0	B1	0.00
ECO129	0	0	0	0	0	B1	2.10
ECO131	0	0	0	0	0	B1	0.00
ECO132	0	0	0	0	1	B1	1.10
ECO134	1	1	1	1	0	B1	1.60
ECO137	1	1	1	1	0	B1	1.60

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS
ECO139	1	1	1	1	0	B1	1.10
ECO142	0	0	0	0	0	B1	1.10
ECO144	0	0	0	0	0	A	1.10
ECO146	0	0	0	0	0	B1	1.10
ECO148	0	1	1	1	0	B1	3.65
ECO151	0	1	1	1	0	B1	2.10
ECO153	0	1	1	0	0	B1	0.95
ECO156	0	0	0	0	0	A	1.10
FECAL4	0	0	0	0	0	A	N.D.
FECAL5	0	0	0	0	0	B1	N.D.
FECAL6	1	1	1	1	1	B1	N.D.
FECAL7	0	1	1	1	1	A	N.D.
FECAL8	0	1	1	1	1	A	N.D.
FECAL9	0	1	1	0	1	D	N.D.
FECAL10	0	1	1	1	1	A	N.D.
FECAL11	0	1	1	0	1	D	N.D.
FECAL12	0	1	1	0	0	B1	N.D.
FECAL13	0	0	0	0	1	A	N.D.
FECAL14	0	1	1	0	1	D	N.D.
FECAL15	1	1	1	1	1	D	N.D.
FECAL17	1	1	1	1	1	A	N.D.
FECAL18	0	0	0	0	1	A	N.D.
FECAL19	0	0	0	0	1	D	N.D.
FECAL20	1	1	1	1	1	B1	N.D.
FECAL21	1	1	1	1	0	D	N.D.
FECAL22	0	1	1	0	0	D	N.D.
FECAL23	0	1	1	0	0	A	N.D.
FECAL24	1	1	1	1	1	D	N.D.
FECAL25	0	1	1	0	1	D	N.D.
FECAL26	0	1	1	0	1	D	N.D.
FECAL27	0	0	0	0	1	D	N.D.
FECAL28	0	1	1	0	1	D	N.D.
FECAL29	0	1	1	0	1	D	N.D.
FECAL30	0	1	1	0	1	D	N.D.
FECAL31	1	1	1	1	1	B2	N.D.
FECAL32	0	1	1	0	1	D	N.D.
FECAL33	0	1	1	0	1	D	N.D.
FECAL34	1	1	1	1	1	D	N.D.
FECAL35	1	1	1	1	1	D	N.D.
FECAL36	0	0	0	0	0	A	N.D.
FECAL37	0	1	1	0	1	D	N.D.

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS
FECAL39	1	1	1	1	1	D	N.D.
FECAL40	1	1	1	1	1	D	N.D.
FECAL41	0	1	1	0	1	D	N.D.
FECAL42	0	1	1	0	1	D	N.D.
FECAL43	0	1	1	0	1	D	N.D.
FECAL44	0	0	0	0	1	B1	N.D.
FECAL45	0	1	1	0	1	D	N.D.
FECAL46	1	1	1	1	0	A	N.D.
FECAL47	0	1	1	0	1	D	N.D.
FECAL49	0	1	1	0	1	D	N.D.
FECAL50	0	0	0	0	0	D	N.D.
FECAL51	0	1	1	0	1	D	N.D.
FECAL52	0	1	1	0	0	B1	N.D.
FECAL53	1	1	1	1	1	D	N.D.
FECAL54	0	1	1	0	0	A	N.D.
FECAL55	0	1	1	0	1	D	N.D.
FECAL56	0	0	0	0	0	A	N.D.
FECAL57	0	1	1	0	1	D	N.D.
FECAL58	0	1	1	0	1	D	N.D.
FECAL59	0	1	1	0	1	D	N.D.
FECAL60	0	0	0	0	0	A	N.D.
FECAL61	0	0	0	0	0	A	N.D.
FECAL62	0	0	0	0	0	A	N.D.
FECAL63	0	0	0	0	1	B2	N.D.
FECAL64	0	0	0	1	0	B1	N.D.
FECAL65	0	0	0	0	1	A	N.D.
FECAL66	0	0	0	0	1	B2	N.D.
FECAL67	0	0	0	0	0	B1	N.D.
FECAL68	0	0	0	0	0	B1	N.D.
FECAL69	0	0	0	0	1	A	N.D.
FECAL70	1	1	1	1	1	A	N.D.
FECAL71	1	1	1	1	1	A	N.D.
FECAL72	1	1	1	1	1	A	N.D.
FECAL73	0	0	0	0	0	A	N.D.
FECAL74	0	0	0	0	0	A	N.D.
FECAL75	0	1	1	0	1	D	N.D.
FECAL76	0	0	0	0	1	B2	N.D.
FECAL77	0	0	0	0	0	A	N.D.
FECAL78	0	1	1	0	1	D	N.D.
FECAL80	1	1	1	1	1	A	N.D.

Strain	*iroN*	*ompT*	*hlyF*	*iss*	*iutA*	Phylogenetic group[1]	median PS
FECAL81	0	0	0	0	1	D	N.D.
FECAL82	0	1	1	0	1	D	N.D.
FECAL83	0	1	1	0	1	D	N.D.
FECAL85	0	0	0	0	0	A	N.D.
FECAL87	1	1	1	1	1	B1	N.D.
FECAL88	0	0	0	0	1	B1	N.D.
FECAL89	0	1	1	0	1	D	N.D.
FECAL 90	0	1	1	0	1	D	N.D.
FECAL 91	0	0	0	0	1	D	N.D.
FECAL 92	1	1	1	1	1	D	N.D.
FECAL 93	1	1	1	1	1	B1	N.D.
FECAL 94	0	0	0	0	1	D	N.D.
FECAL 95	0	1	1	0	1	D	N.D.
FECAL 96	0	1	1	0	1	D	N.D.
FECAL 97	0	0	0	0	1	D	N.D.
FECAL 98	1	1	1	1	1	B1	N.D.
FECAL99	0	0	0	0	1	D	N.D.
FECAL100	0	0	0	0	0	A	N.D.
FECAL101	0	0	0	0	0	D	N.D.
FECAL102	0	0	0	0	1	B1	N.D.
FECAL103	0	1	1	0	1	D	N.D.
FECAL104	0	1	1	0	1	D	N.D.
FECAL105	0	1	1	0	1	D	N.D.
FECAL106	0	0	0	0	1	A	N.D.
FECAL107	0	0	0	0	1	B1	N.D.
FECAL108	0	1	1	0	0	B1	N.D.
FECAL109	0	1	1	0	0	B1	N.D.
FECAL110	0	0	0	0	0	B1	N.D.
FECAL111	0	0	0	0	1	A	N.D.
FECAL112	1	1	1	1	1	D	N.D.
FECAL113	0	0	0	0	1	A	N.D.
FECAL114	0	0	0	0	1	A	N.D.
FECAL115	0	0	0	0	1	D	N.D.
FECAL116	0	0	0	0	1	A	N.D.
FECAL117	0	0	0	0	0	A	N.D.
FECAL118	0	0	0	0	1	D	N.D.

1 The phylogenetic grouping and the PS values of the APEC strains (PR and RS) were adopted from previous publications (Barbieri*et al*., 2013; Barbieri*et al*., 2014).

Analysis of prevalence, gene expression and functionality of Type 6 Secretion System in APEC strains

Aline L. de Oliveira, Nicolle L. Barbieri, Felipe Ricachenewsky, Janete Mariza Adamski, Roberta S. dos Reis, Fabiana Horn*

Departamento de Biofísica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil;

*Corresponding author:

Av. Bento Gonçalves, 9500

Porto Alegre/ RS - Brasil

P.O. Box 15005

91501-970

Tel: +55 51 3308 7610

e-mail: fabiana.horn@ufrgs.br

ABSTRACT

Type Six Secretion System (T6SS) has been recently described and it was reported to be associated with pathogenesis, bacterial competition and antibacterial properties of various bacterial species. T6SS was recently associated with pathogenicity of APEC strains, and it was also shown that an APEC strain may harbor up to three clusters of T6SS in its genome. In this work we analyzed the frequencies of two copies of T6SS encoding genes *clpV*, *vgrG*, *icmF* and *dotU* in a Brazilian collection of *E. coli* isolated from birds with colibacillosis (APEC). We found that the frequencies of *clpV* and *vgrG* (both 1 and 2) are higher than the frequencies of *icmF* and *dotU*, and that some of the APEC isolates analyzed here harbor two copies of one or more of these genes. We also observed that some of the *vgrG*$^{+}$ strains presented resistance to predation by *Dictyostelium discoideum*, a feature related to T6SS. We also analyzed, in some T6SS genes positive strains, the expression of *vgrG* (1 and 2) and *clpV* when grown in culture medium. T6SS genes expression and protein secretion by APEC O1 strain were analyzed both in pure culture and in contact with eukaryotic cells.

Keywords: APEC, Type Six Secretion System, T6SS, pathogenicity.

1. INTRODUCTION

Avian pathogenic *Escherichia coli* (APEC) are the etiological agent of colibacillosis, causing respiratory and systemic infections and financial losses in the poultry industry worldwide (Dho-Moulin e Fairbrother, 1999). The APEC pathotype is not already defined, but many virulence-associated genes (VAGs) had been reported to be associated with pathogenesis of these strains (Dziva e Stevens, 2008) . In addition to VAGs, other factors may be associated to bacterial pathogenesis, like the bacterial Secretion Systems, protein machineries responsible for bacterial protein secretion to the external milieu or straight into the host cells (Tseng *et al.*, 2009).

Six secretions systems (1-6) have been described for gram-negative bacteria so far. Secretion systems 2 and 5 are *Sec*-dependent, which means that they secrete bacterial proteins from the periplasmic space to the extracellular medium after these proteins have been translocated to periplasmic space by *Sec* systems. Secretion systems 1, 3, 4 and 5, in turn, secrete bacterial proteins across the inner and outer membranes of bacteria, and inject these proteins directly into the cytosol of the host cell in a one-step process (Tseng *et al.*, 2009).

The Type 6 Secretion System, described by Pukatzki *et al*., is the most recent protein secretion system described for gram-negative bacteria. It was first described in *Vibrio cholera* strain V52, and is associated with the capacity of escaping from predation by the amoebae *Dyctiostelium discoideum*, an eukaryotic cell that mimics mammalian macrophages (Pukatzki *et al.*, 2006). Although T6SS has been recently described, clues of its existence are not that recent. In 1996, Hcp (hemolysin co-regulated protein) from V. *cholerae* was shown to be secreted without signal peptide cleavage, differently from other V. *cholerae* secreted proteins (Williams *et al.*, 1996).

Candidates for Type 6 Secretion gene *loci* were first *in silico* analyzed in 2003. These *loci* were characterized by encoding a protein associated with Type 4 Secretion System in *Legionella peumophila*, called IcmF. However, most protein encoded by these *loci* did not seem to be homologous to the proteins of the other

known secretion systems. Then, this cluster was called IAHP (IcmF-associated homologous protein) (Das e Chaudhuri, 2003). These and other findings were important for the identification of Type 6 secretion in 2006.

By 2008, type 6 secretion had been described in *V. cholerae*, *Pseudomonas aeruginosa* and *Burkholderia mallei* (Pukatzki *et al.*, 2006; Mougous *et al.*, 2007; Schell *et al.*, 2007). Eighteen genes have been described in *Vibrio cholera* T6SS, 4 of which are thought to be structural components, 2 are effector molecules and 1 is a chaperone (Pukatzki *et al.*, 2006b). Using bioinformatics, Shrivastava and Mande have searched for orthologous of T6SS components of *V. cholerae*, *P. aeruginosa* and *B. mallei* in bacterial species. They found that 42 bacterial species, including avian pathogenic *E. coli* (APEC) and uropathogenic *E. coli* strains CFT073, UTI189 and 536 carry orthologs of at least 10 genes of T6SS (Shrivastava e Mande, 2008).

The importance of T6SS in pathogenesis has been shown in diverse bacterial species. In *B. mallei*, a human and animal pathogen, T6SS is important for the proliferation inside macrophages, and the expression of a Hcp-related protein is induced during *in vivo* infection (Schell *et al.*, 2007). *Edwardsiella tarda*, a fish pathogen, has an active T6SS (Zheng e Leung, 2007), and in *P. aeruginosa*, Hcp1 is secreted by clinical isolates, indicating that the system is active during infection (Mougous *et al.*, 2006).

In addition to its association with pathogenicity, T6SS has been related to other bacterial traits. T6SS has been reported to be involved in the antibacterial property of *V. cholerae* towards Gram-negative pathogens like *Salmonella Typhimurium*, *Citrobacter rodentium* and *Pseudomonas aeruginosa* (Macintyre *et al.*, 2010). T6SS has also been shown to be involved in bacterial competition in *Pseudomonas fluorescens* (Decoin *et al.*, 2014).

In 2010, de Pace et al. showed the influence of T6SS core genes *clpV* and *hcp* on the pathogenicity of an APEC strain (De Pace, F. *et al.*, 2010). In recent works, comparative genomics showed that APEC strains may harbor three T6SS *loci* (Ma *et al.*, 2013), and that a specific APEC strain harbors two functional T6SS involved in different pathogenic pathways (Ma *et al.*, 2014).

In this work, we show the frequencies of two copies of T6SS genes *icmF*, *dotU*, *clpV* and *vgrG*. We have also assessed the capacity of some *vgrG*$^{+}$ strains to resist predation by *D. discoideum* amoebae*,* and the expression of *vgrG* and *clpV* genes by some APEC strains under different growth conditions. Furthermore, we assessed the protein secretion of APEC O1 strain in the culture medium or in contact with eukaryotic cells.

2. MATERIAL AND METHODS

2.1. Bacterial strains

We evaluated 187 APEC strains, including 137 isolates from cellulitis lesions (PR) and 50 isolates obtained from different organs (skin, air sacs, liver, heart, and intestine) from broiler chickens with signs of colisepticemia (RS). These isolates have been previously characterized regarding pathogenicity score, prevalence of virulence-associated genes and phylogeny (Barbieri *et al.*, 2013; Barbieri *et al.*, 2014). APEC O1 strain was used as basis for primer design and positive control in PCR reactions.

2.2. DNA extraction

Strains were streaked on LB (Luria-Bertani) agar and grown at 37 °C overnight. A loop of colonies was suspended in 200 µL of milli-Q water, incubated at -20 °C for 10 min followed by 10 min at 100 °C, and centrifuged at 14,000 *g* for 10 min. The supernatants were used as templates for PCR and stored at 4 °C.

2.3. Oligonucleotides for detection of of *clpV*, *vgrG*, *icmF* and *dotU*

Primers were designed using Primer3 Software based on the sequences of the following ExPEC: APEC strains APEC O1 (NC_008563.1), SCI-07 - T6SS1 contig03 (NZ_AJFG01000003.1) and T6SS2 contig26 (NZ_AJFG01000026.1) - and SEPT362 - T6SS 1 contig04 (NZ_AOGL01000004.1) and T6SS 2 contig55 (NZ_AOGL01000055.1) and contig56 (NZ_AOGL01000056.1) - UPEC strains 536 (NC_008253.1); UTI89 (NC_007946.1); and CFT073 (NC_004431.1), and the NMEC strain s88 (NC_011742.1).

We designed two pairs of primers for each gene, since these genes may be present in two or more different copies in the genome. We performed *in silico* PCR using pDRAW Software to test the annealing of the primers to the APEC O1 genome. The identity of the amplicon was assessed using BLAST (Basic Local Alignment Search Tool). The sequences of primers are listed in Supplementary Table 1.

2.4. Genotyping of APEC strains by conventional PCR

Reactions were performed in a GenePro Thermal Cycler (Bioer Technology, China) as follows: denaturation for 3 min at 94 °C; 25 cycles of 30 s at 94 °C, 30 s at 58 °C and 1 min at 72 °C, followed by a final extension step of 5 min at 72 °C. PCR products were analyzed by gel electrophoresis, and the sizes of amplicons were determined by comparison with a 100-bp molecular weight marker (Ludwig Biotec, Porto Alegre, Brazil). APEC O1 strain was used as positive control.

Reactions were performed in a final volume of 25 µL (in milli-Q water), containing 2,5 µL of 10x *Taq* polimerase Buffer; 2,0 µL of 50 mM $MgCl_2$; 2 U *Taq* DNA polimerase; 0,5 µL of 10mM dNTP mix; 20 pmol of oligonucleotides and 4 µL of DNA.

2.5. Plaque assay

Plaque assay was performed according to Pukatzki et al. (Pukatzki *et al.*, 2006) with slight modifications. Bacteria were grown in LB overnight, pelleted by centrifugation, washed once, and resuspended in PBS. *Dictyostelium discoideum* cells cultured in SM broth (glucose 10 g/L; proteose peptone 10 g/L; yeast extract 1 g/L; magnesium sulfate heptahydrate 1 g/L; monobasic potassium phosphate 1.9 g/L; bibasic potassium phosphate 0.6 g/L; pH 6.2) were collected by centrifugation, washed once with PBS, and added to the bacterial suspensions at a final concentration of 2-5 x 10^3 *D. discoideum* cells per mL bacterial suspension (10^8 CFU); 0.5 – 1.0 mL of this mixture was plated on SM/5 (glucose 2 g/L; bacto peptone 2 g/L; yeast extract 0.2 g/L; magnesium sulfate heptahydrate 0.1 g/L; monobasic potassium phosphate 1.9 g/L; bibasic potassium phosphate 1.0 g/L; agar 15 g/L; pH 6.5) plates and allowed to dry under a sterile air flow. Plates were incubated in at 22 °C in the dark. Plates were analyzed 3 and 5 days after incubation for plaque formation by *D.discoideum* and photographed on a transilluminator.

2.6. RNA extraction

To assess the expression of *vgrG* and *clpV* genes, *E. coli* strain APEC O1 was grown overnight at 37 °C in 200 mL of LB broth or DMEM media statically or under shaking. Strains SEPT 362, PR011 and PR014 and PR025 were grown overnight at 37 °C in 200 mL DMEM statically. RNA of APEC O1 was also extracted after two hours of contact with J774 or HeLa cells to verify if cellular contact induces T6SS expression (bacterium-cell contact experiments are described below). Two biological replicates of each sample were prepared.

RNA from these strains was isolated using Quickzol® (Ludwig Biotec) according to the manufacturer's instructions. Briefly, the bacterial cells were pelleted by centrifugation at 4000 *g* for 10 min at 4 °C. One milliliter of Quickzol® was added to the pellet, followed by incubation at room temperature for 5 min. Chloroform was then added in a ratio of 200 µl of chloroform:1 mL of Quickzol®. The solution was mixed and incubated at room temperature for 10 min. Then, it was centrifuged at 12000 *g* for 15 min at 4 °C. The aqueous phase was then transferred to a clean tube and it was added an equal volume of isopropanol. The sample was incubated at room temperature for 10 min and then centrifuged at 12000 *g* for 10 min at 4 °C. The supernatant was discarded and the pellet was washed with 1 mL of 75% ethanol, and centrifuged at 7500 *g* for 5 min at 4 °C. Supernatant was discarded and the pellet was resuspended in 25 µl of sterile milli-Q water. The concentration of RNA was determined using a Spectrophotometer (ND-1000) (NanoDrop). When the RNA concentration was too high, the sample was diluted in milli-Q water.

2.7. DNase treatment of RNA and synthesis of the first strand of cDNA

Treatment of 1 µg RNA was performed as follows: 2 U DNase Turbo, 1 µl 10x DNase reaction buffer, and sterile milli-Q water (to 10 µl) were added to 0.5 mL tubes containing 1 µg RNA. The mix was incubated at 37 °C for 30 min. Then, 1 µl of 25 mM EDTA was added to each tube, and the mix was incubated at 75 °C for 10 minutes. The DNase-treated RNA was stored at -20°C until use.

One microgram of DNase-treated RNA from each biological replicate was reverse transcribed as follows: 500 ng of random oligonucleotide hexamers (0.5

µg/µL) (Promega) and 1 µl of 10 mM dNTP were added to each tube with 1 µg of DNase-treated RNA. The mix was incubated at 65 °C for 5 minutes. Then, 2 µl of 10 mM DTT and 4 µl of 5x first-strand buffer were added to each tube. After incubation at 37 °C for 2 minutes, 1 µl of reverse transcriptase M-MLV (200 U/µl) was added to each tube, and the mix was incubated at 37 °C for 50 min and then at 70 °C for 15 min. cDNA samples were stored at 20°C until use.

2.8. Quantitative RT-PCR

Quantitative real-time RT-PCR (qRTPCR) was performed on a Step One™ Real Time PCR System (Applied Biosystems). Primers for qRT-PCR (Supplementary Table 1) were purchased from IDT DNA Technologies. cDNA was diluted 1:20, for use in qRT-PCR. Each reaction was performed in a final volume of 20 µl containing 25 ng cDNA, 2 µl *Taq* DNA polimerase buffer, 1.25 µl $MgCl_2$ 50 mM, 0,2 dNTP 10 mM, 0,4 of each primer (10 mM), 2 µl of diluted (1:100) Sybr® Green (Invitrogen™) and 0,05 µl Platinum® *Taq* DNA polimerase (Invitrogen™). PCR conditions were as follows: 95 °C for 20 sec, 40 cycles at 95 °C, 57 °C and 72 °C for 3 sec, 30 sec and 15 sec respectively, 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec.

Threshold fluorescence was established within the geometric phase of exponential amplification, and the cycle threshold (CT) was determined for each sample RNA. The CT from each replicate was averaged. Expression levels were normalized using the housekeeping gene *tus*, which encodes a DNA replication terminus site-binding protein, as endogenous control. Melting curve analyses were performed after each reaction to ensure amplification specificity. Differences (*n*-fold) in transcripts were calculated using the relative comparison method (Schmittgen *et al.*, 2000).

2.9. Protein secretion analysis

We assessed protein secretion of APEC O1 according to Decoin *et. al*. (Decoin *et al.*, 2014), with slight modifications. First, APEC O1 was grown in 5 mL of LB in a 250 mL Erlenmeyer flask under shaking at 180 rpm overnight. Then, the

supernatant was harvested by centrifuging the culture for 10 min at 4 °C and passing it through a 0.22 µm membrane. TCA was added to the supernatant to a final concentration of 10% and incubated at 4 °C for 24-48 hours. Supernatant was removed by centrifugation at 13000 *g* for 30 minutes at 4 °C. The protein pellet was washed twice with 5 mL of 20 mM Tris base in cold acetone and centrifuged at 13000 *g* for 30 minutes at 4 °C. The dry pellet was then resuspended in milli-Q water. Proteins were quantified in a NanoDrop Spectrophotometer (Thermo Scientific), and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Briefly, 200 µg of protein were mixed with 5 µL of the sample buffer, boiled for 5 min and then cooled to room temperature before loading. The electrophoresis was run for 30 min at 80 V followed by 90 min at 120 V. Broad Range Protein Molecular Weight Markers (Promega) was used as reference for molecular weight of proteins analyzed. The protein secretion was also assessed with bacteria grown in DMEM as well as in contact with eukaryotic cells J774 (murine macrophage) and HeLa (human epithelial cells).

2.10. Bacterium-cell contact assays

We have also investigated gene expression and protein secretion of APEC O1 when in contact with eukaryotic cells J774 and HeLa. The bacterium-cell contact assays were performed in duplicate, as follows.

Briefly, eukaryotic cells (J774 or HeLa) were growth in DMEM. The cells were layered, at a concentration of 10^6 cells/well, on 12-well plates and incubated at 37 °C under a humidified atmosphere containing 5% CO_2. These cells were allowed to grow for 2 days before use. Before infection, cells were washed with PBS and resuspended in fresh cell culture medium. Two hundred µl of overnight culture of APEC O1 growth statically in DMEM were added to each well. The plate was incubated for 2 hours at 37 °C under a humidified atmosphere containing 5% CO_2. Then, the cell culture supernatant containing bacteria was collected and centrifuged at 4000 *g* for 10 minutes at 4°C. The bacterial supernatant was collected and passed through a 0.22 µm membrane for use for protein isolation as

described above (protein secretion analysis). The bacterial pellet was used for RNA isolation as described above.

2.11. Recombinant DNA Techniques

Polymerase chain reaction (PCR), DNA ligation, electroporation and DNA gel electrophoresis were performed according to Sambrook and Russel (Sambrook e Russell, 2001) unless otherwise stated. The oligonucleotides used for this purpose were purchased from Integrated DNA Technologies (IDT) and are listed in Supplementary Table 1. Restriction and DNA-modifying enzymes were used according to supplier's recommendation. Plasmids were isolated using Wizard® *Plus* SV Minipreps DNA Purification Systems (Promega). Plasmids, PCR products and restriction fragments were purified using Wizard® SV Gel and PCR Clean-up System (Promega). For conjugation, we used APEC O1 *ΔlacZ* as recipient and *E. coli* S17 as donor.

3. RESULTS

3.1. Genotyping of APEC strains for *clpV*, *vgrG*, *icmF* and *dotU* genes

We screened 187 avian pathogenic *E. coli* isolates (50 colisepticemic isolates – RS – and 137 cellulitis isolates – PR) for the presence of two copies of *clpV*, *vgrG*, *icmF* and *dotU* genes. The frequencies of the four genes, separated in two sets, in both classes of isolates is shown in Tables 1 (T6SS1) and 2 (T6SS2).

From all isolates (187), 12 presented two copies of *icmF*, 20 presented two copies of *dotU*, 37 presented the two copies of *clpV*, and 65, the two copies of *vgrG*. The results for the genotyping are shown in Supplementary Table 2.

We did not find a positive correlation between the presence of these genes and the previously established pathogenicity score of these strains (data not shown).

3.2. Plaque assay

The free-living amoeba *D. discoideum* was used as a model of eukaryotic cell that mimics the mammalian macrophage since it interacts with microbes and presents phagocytic feeding behavior. Pukatzki *et al*. have shown that *Pseudomonas aruginosa* is able to kill the amoeba *D. discoideum* through the delivery of cytotoxic ExoU protein by T3SS (Pukatzki *et al.*, 2002). In a further work, they have also shown that a *Vibrio cholerae* strain V52 was able to kill the amoebae and that this phenotype was due to a gene cluster that encodes a distinct secretion system, which they designated T6SS (Pukatzki *et al.*, 2006).

To test whether our APEC strains could display the phenotype of resisting predation by *D. discoideum*, we performed the plaque assay with some of the isolates that contain at least one copy of *vgrG*. We tested 31 APEC isolates (PR002 – PR034), in addition to SCI-07, SEPT 362, and APEC O1 strains searching for an association of the presence of *vgrG* with resistance to predation and pathogenicity score. We chose cellulitis isolates because they have higher median pathogenicity scores (PS) than colisepticemia isolates.

We found three different patterns among the tested isolates: susceptible to predation (larger plaques); intermediate (small plaques) and resistant to predation (no plaques) (Figure 1). In this classification, we took into account only the presence and size of plaques. The formation of plaques indicates that *D. discoideum* was able to phagocyte the bacterium. The absence of plaques indicates that the bacterium was able to resist predation by *D. discoideum*, preventing the growth of the amoebae or even killing it.

From the 31 isolates tested, 18 were susceptible, 5 were intermediate, and 8 were resistant to predation by *D. amoebae* according to our criteria.

Although we found some apparently resistant isolates, we cannot affirm that this phenotype was due to a functional T6SS, since we did not test the expression of T6SS genes nor the secretion of T6SS-secreted proteins under this condition.

3.3. qRT-PCR

We selected three samples from our APEC collection to analyze the expression of *vgrG1*, *vgrG2* and *clpV2* genes by qRT – PCR. These samples were selected according to the presence of the aforementioned genes detected by conventional PCR, the phenotype that they presented towards *D. discoideum*, and their pathogenicity scores previously determined by Barbieri *et al*. (Barbieri *et al.*, 2013). The strains tested were: PR011 (resistant to predation by *D. discoideum* and high PS); PR014 (susceptible to predation by *D. discoideum* and low PS); PR025 (susceptible to predation by *D. discoideum* and high PS); APEC O1 (the same strain used as template for primers design; resistant to predation); and SEPT 362 (an APEC strain whose virulence was shown to be influenced by T6SS). The strains were grown in DMEM statically overnight. Only APEC O1 was grown both in DMEM or LB broth, and both statically and under shaking. All experiments were performed in biological duplicates and technical triplicates. The housekeeping gene *tus*, that encodes a DNA replication terminus site-binding protein, was used as endogenous control for data normalization. Contrary to our expectations, we did not observed significant differences between the expression of *vgrG1*, *vgrG2* and *clpV2* genes by these strains. PR011, PR014 and PR025 did not show any

expression of *clpV2*, so this data was suppressed from the figure. So, we wondered whether the system could be activated by contact with eukaryotic cells, as observed in other bacterial species. We used APEC O1 as a model and performed bacteria-cell contact assays as described in materials and methods, using HeLa and J774 cells. Then we analyzed the expression of *vgrG1*, *vgrG2* and *clpV2* by APEC O1 after two hours of contact with HeLa or J774. These experiments were also performed in biological duplicates and technical triplicates. We compared these results with that obtained for APEC grown in culture medium and no significant difference was found in the expression of *vgrG1*, *vgrG2* and *clpV2* in APEC O1 comparing the two conditions (Figure 2).

3.4. Protein Secretion

We analyzed the protein secretion by APEC O1 under different conditions: APEC O1 grown in LB medium under shaking at 180 rpm overnight; APEC O1 grown statically in LB medium overnight; APEC O1 after two hours of contact with HeLa cells and APEC O1 after two hours of contact with murine macrophages J774. As negative controls for bacterial secreted proteins, we used the supernatant of non-infected HeLa and J774 cultures and proceed with the method for protein isolation. Proteins were quantified in a NanoDrop Spectrophotometer (Thermo Scientific), and separated by SDS-PAGE. We found different patterns of protein secretion under the different conditions tested (Figure 3). The identification of these proteins will be assessed by Mass Spectrometry.

3.5. Construction of beta-gal transformants

We used recombinant DNA techniques to construct beta-gal transformant strains of APEC O1 in order to measure the expression of *clpV*, *vgrG*, *icmF* and *dotU* through beta-galactosidase activity. Having a transformant allow us to assess gene expression under various conditions in a faster way compared to qRT-PCR. To date, we have constructed the transformants APEC O1 *ΔlacZdotU1*, APEC O1 *ΔlacZdotU2* and APEC O1 *ΔlacZ clpV1*. The transformants for *clpv2*, *icmF1*, *icmF2*, *vgrG1* and *vgrG2* genes are in progress. Once we have all the

transformants, we will perform beta-galactosidase activity assays to assess gene expression under different conditions.

4. DISCUSSION

Type 6 Secretion system has been associated with pathogenesis of diverse bacterial species, including pathogenicity of avian pathogenic *Escherichia coli* strains (De Pace, Fernanda *et al.*, 2010). The screening of our APEC collection for *clpV*, *vgrG*, *dotU* and *icmF* showed that some strains possess two copies of *icmF*, *dotU* and *clpV*, and almost 35% presented the two copies of *vgrG*. This is in agreement with previous works that showed that APEC strains may harbor up to three clusters of T6SS (Ma *et al.*, 2013).

The fact that some of our strains carry T6SS in their genomes does not necessarily mean that the system is assembled and functional.

Pukatzki *et al*. have shown that T6SS confers to *V. cholerae* strain V52 the ability to resist predation by *D. discoideum* (Pukatzki *et al.*, 2006), which is a model of eukaryotic cell whose phagocytic properties are very similar to macrophages. Thus, to verify if APEC strains that carry the four screened T6SS genes assemble a functional system, we assessed the ability of these strains to resist predation by *D. discoideum*. We observed that some of these strains were indeed able to resist predation, indicating that T6SS may be active under contact with amoebae. Nevertheless, despite the observed phenotype, we cannot affirm that it is due to an active T6SS. More detailed analyses of the genes expressed and proteins secreted under contact with *D. discoideum* would be necessary to assess the involvement of T6SS in the resistance to predation displayed by our strains.

In an attempt to relate the phenotype described above with the expression of T6SS, we performed gene expression analysis by qRT-PCR of *vgrG* (1 e 2) and *clpV2*. We chose three samples (PR11, PR14 and PR25) from our APEC collection, based on their phenotype and pathogenicity score. We expected that a strain with higher PS and resistant to predation would display a higher level of expression of the genes than a strain with lower PS and susceptible to predation. However, we did not find significant difference of gene expression between the strains analyzed when grown in culture medium only. In 2010, De Pace *et al*. (De Pace, F. *et al.*, 2010) have shown that the APEC strain SEPT362 expressed *hcp*

and *clp*V after contact with HeLa cells. Given that, we assessed the expression of *vgrG* (1 e 2) and *clpV2* by APEC O1 after 2 hours of contact with HeLa cells or J774 murine macrophages. Contrary to our expectations, we did not observe an increase of *vgrG* and *clpV* expression under these conditions. Maybe the time of contact was not long enough to assess the expression of these genes.

The release of VgrG and Hcp proteins is indicative of a functional T6SS (Pukatzki *et al.*, 2009). In 2014, Decoin *et al*. showed that *Pseudomonas fluorescens* MFE01 was able to secrete these proteins to culture medium at 28 ºC, showing that this strain expressed a T6SS constitutively (Decoin *et al.*, 2014). In this work, we assessed protein secretion by APEC O1 in culture medium or in contact with HeLa or J774 cells. We separated these proteins by SDS-PAGE, and we observed different patterns of secretion comparing bacteria grown in culture medium with bacteria submitted to contact with eukaryotic cells. The proteins isolated in both conditions will be sent to analysis by Mass Spectrometry. If one of the T6SS effectors is found in the pool of secreted proteins, we will be able to affirm that APEC O1 expresses a functional T6SS. Once we have this data, it will be easier to study the influence of this system on the virulence of APEC O1 and other APEC strains.

REFERENCES

Barbieri N.L., de Oliveira A.L., Tejkowski T.M., et al. Genotypes and pathogenicity of cellulitis isolates reveal traits that modulate APEC virulence. PLoS One 2013; 8:e72322.

Barbieri N.L., Oliveira A.L., Tejkowski T.M., et al. Molecular characterization and clonal relationships among *Escherichia coli* strains isolated from broiler chickens with colisepticemia. Foodborne Pathogens and Disease 2014; accepted for publication.

Das S. and Chaudhuri K. Identification of a unique IAHP (IcmF associated homologous proteins) cluster in *Vibrio cholerae* and other proteobacteria through in silico analysis. In Silico Biology 2003; 3:287-300.

de Pace F., Nakazato G., Pacheco A., de Paiva J.B., Sperandio V. and da Silveira W.D. The type VI secretion system plays a role in type 1 fimbria expression and pathogenesis of an avian pathogenic *Escherichia coli* strain. Infection and Immunity 2010a; 78:4990-4998.

de Pace F., Nakazato G., Pacheco A., de Paiva J.B., Sperandio V. and da Silveira W.D. The type VI secretion system plays a role in type 1 fimbria expression and pathogenesis of an avian pathogenic *Escherichia coli* strain. Infection and Immunity 2010b; 78:4990-8.

Decoin V., Barbey C., Bergeau D., et al. A Type VI Secretion System Is Involved in *Pseudomonas fluorescens* Bacterial Competition. PLoS One 2014; 9:e89411.

Dho-Moulin M. and Fairbrother J.M. Avian pathogenic *Escherichia coli* (APEC). Veterinary research 1999; 30:299-316.

Dziva F. and Stevens M.P. Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathology 2008; 37:355-366.

Ma J., Bao Y., Sun M., et al. Two functional type VI secretion systems in avian pathogenic *Escherichia coli* are involved in different pathogenic pathways. Infect Immun 2014; 82:3867-79.

Ma J., Sun M., Bao Y., et al. Genetic diversity and features analysis of type VI secretion systems loci in avian pathogenic *Escherichia coli* by wide genomic scanning. Infection, genetics and evolution: journal of molecular epidemiology and evolutionary genetics in infectious diseases 2013; 20:454-64.

MacIntyre D.L., Miyata S.T., Kitaoka M. and Pukatzki S. The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. Proc Natl Acad Sci U S A 2010; 107:19520–19524.

Mougous J.D., Gifford C.A., Ramsdell T.L. and Mekalanos J.J. Threonine phosphorylation post-translationally regulates protein secretion in *Pseudomonas aeruginosa*. Nature Cell Biology 2007; 9:797-803

Pukatzki S., Kessin R.H. and Mekalanos J.J. The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. Proc Natl Acad Sci U S A 2002; 99:3159-64.

Pukatzki S., Ma A.T., Sturtevant D., et al. . Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proceedings of the National Academy of Sciences of the United States of America 2006a; 103:1528-1533.

Pukatzki S., Ma A.T., Sturtevant D., et al. . Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the Dictyostelium host model system. Proc Natl Acad Sci U S A 2006b; 103:1528-33.

Pukatzki S., McAuley S.B. and Miyata S.T. The type VI secretion system: translocation of effectors and effector-domains. Curr Opin Microbiol 2009; 12:11-7.

Sambrook J. and Russell D.W. *Molecular Cloning: A Laboratory Manual*. 2001.

Schell M.A., Ulrich R.L., Ribot W.J., et al. . Type VI secretion is a major virulence determinant in *Burkholderia mallei*. molecular Microbiology 2007a; 64:1466-85.

Schmittgen T.D., Zakrajsek B.A., Mills A.G., Gorn V., Singer M.J. and Reed M.W. Quantitative reverse transcription-polymerase chain reaction to study mRNA decay: Comparison of endpoint and real-time methods. Analytical Biochemistry 2000; 285:194-204.

Shrivastava S. and Mande S.S. Identification and Functional Characterization of Gene Components of Type VI Secretion System in Bacterial Genomes. Plos One 2008; 3.

Tseng T.-T., Tyler B.M. and Setubal J.C. Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. Bmc Microbiology 2009; 9.

Williams S.G., Varcoe L.T., Attridge S.R. and Manning P.A. *Vibrio cholerae* Hcp, a secreted protein coregulated with HlyA. Infection and Immunity 1996; 64:283-289.

Zheng J. and Leung K.Y. Dissection of a type VI secretion system in *Edwardsiella tarda*. Molecular Microbiology 2007; 66:1192-206.

LEGENDS TO FIGURES

Figure 1. Plaque assay. *D. discoideum* cells were plated on SM/5 with APEC strains at a density of 2-5 x 10^3 amoeba per plate. The APEC strains were considered susceptible (A – PR020), intermediate (B – PR016) or resistant (C– PR024) to predation according to the presence and size of plaques.

Figure 2. Expression of *vgrG1*, *vgrG2* and *clpV2* genes. Real time quantitative RT-PCR was used to analyze the expression of *vgrG1*, *vgrG2* and *clpV2* genes by SEPT 362, APEC O1, PR011, PR014 and PR025. ΔCT reflects the gene expression normalized by the expression of housekeeping gene *tus*. Data represent the average of two experiments done in triplicate.

Figure 3. SDS-PAGE of the proteins secreted by APEC O1. Lane 2: MW - Molecular weight marker; Lane 4: Proteins secreted by APEC O1 after two hours of contact with J774 cells; Lanes 5 and 6: Proteins secreted by APEC O1 grown in LB broth at 37 ºC overnight.

FIGURES

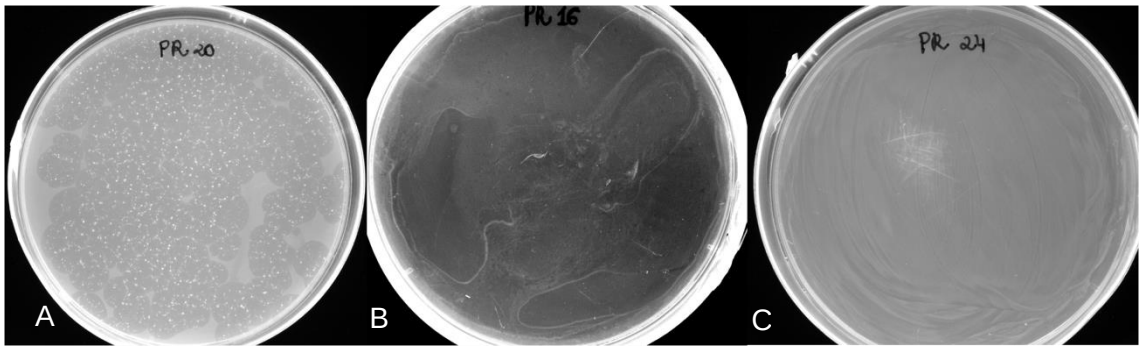


Figure 1. Plaque assay. *D. discoideum* cells were plated on SM/5 with APEC strains at a density of 2-5 x 10^3 amoeba per plate. The APEC strains were considered susceptible (A – PR020), intermediate (B – PR016) or resistant (C– PR024) to predation according to the presence and size of plaques.

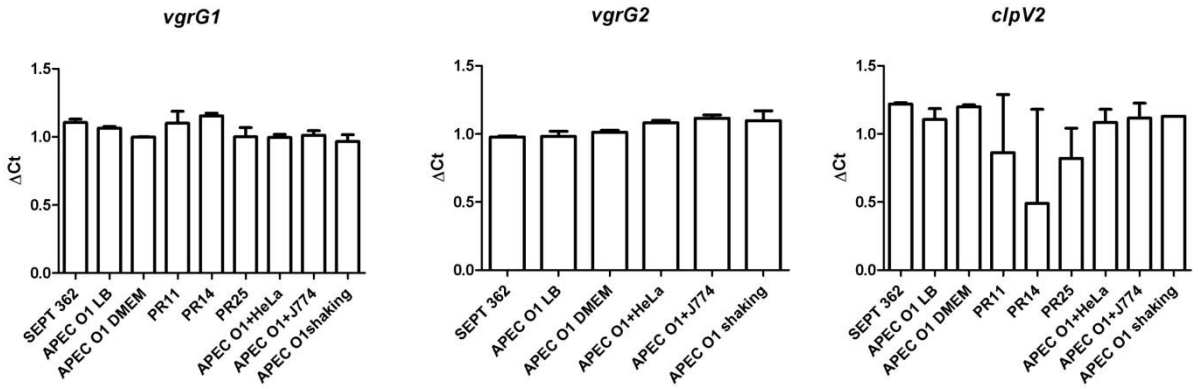


Figure 2. Expression of *vgrG1*, *vgrG2* and *clpV2* genes. Real time quantitative RT-PCR was used to analyze the expression of *vgrG1*, *vgrG2* and *clpV2* genes by SEPT 362, APEC O1, PR011, PR014 and PR025. ΔCT reflects the gene expression normalized by the expression of housekeeping gene *tus*. Data represent the average of two experiments done in triplicate.

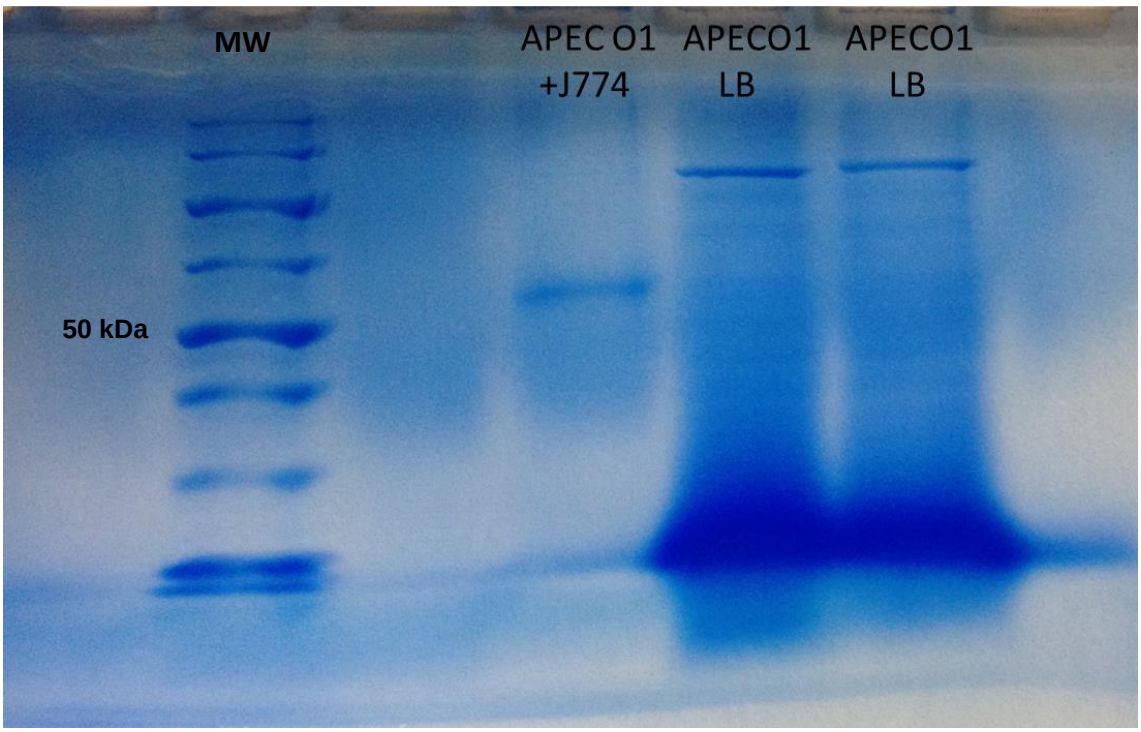


Figure 3. SDS-PAGE of the proteins secreted by APEC O1 under different conditions. Lane 2: MW - Molecular weight marker; Lane 4: proteins secreted by APEC O1 after two hours of contact with J774 murine macrophages; Lanes 5 and 6: proteins secreted by APEC O1 grown in LB broth at 37 ºC overnight.

Table 1. Frequencies of T6SS1 *clpV*, *vgrG*, *icmF* and *dotU* genes in APEC isolates

Gene	Colisepticemia (RS) (n=50)	Cellulitis (PR) (n=137)	Total (n=187)
clpV	25 (50%)	55 (40%)	80 (43%)
vgrG	44 (88%)	102 (74%)	146 (78%)
icmF	0	30 (22%)	30 (16%)
dotU	0	31 (23%)	31 (16.5%)

Table 2. Frequencies of T6SS2 *clpV*, *vgrG*, *icmF* and *dotU* genes in APEC isolates

Gene	Colisepticemia (RS) (n=50)	Cellulitis (PR) (n=137)	Total (n=187)
clpV	15 (30%)	90 (66%)	105 (56%)
vgrG	15 (30%)	89 (65%)	104 (55%)
icmF	9 (18%)	55 (40%)	64 (34%)
dotU	10 (20%)	66 (48%)	76 (41%)

Supplementary Table 1. Oligonucleotide sequences used as PCR primers

Conventional PCR

Name of sequence	Sequence	Amplicon size
icmF1for	5'GCTGGACGGTGAGAACATGGATG3'	482 bp
icmF1rev	5'CGTGATCGCGATAATCACCATA3'	
dotU1for	5'ACAGTCGCTGCTGGTTCATTT3'	314 bp
dotU1rev	5'CATGCTTGATGGTCAGTCGTC3'	
clpV1for	5'CTGCATAAGCATCTACTGAC3'	400 bp
clpV1rev	5'CGATGTTTTTACAGTCAATC3'	
vgrG1for	5'GTATCTTCCAGAATGAGGAC3'	831 bp
vgrG1rev	5'CATGTTCATCACAGAAGATT3'	
icmF2for	5'GCTGTAATGCTGGAAGCACA3'	515 bp
icmF2rev	5'ATACGGTATTGCAGGCGTTC3'	
dotU2for	5'CGGATGAAGCAGGTGCTACAG3'	320 bp
dotU2rev	5'AGCAGTTCATCCACCAGTACA3'	
clpV2for	5'CATATGCTGGACAGCGCCATT3'	282 bp
clpV2rev	5'TTCTGCCTGTAGCTCATCACGC3'	
vgrG2for	5'GCACCAGTTCACTGCTGATG3'	170 bp
vgrG2rev	5'GTAGCCGTAGCGTTCTGAGTA3'	

qRT – PCR

Name of sequence	Sequence	Amplicon size
vgrG1for	5'ATATCCACGCGCAGAAGAAC3'	110 bp
vgrG1rev	5'CCCCACCGTTATCTTCTGGT3'	

qRT - PCR

Name of sequence	Sequence	Amplicon size
vgrG2for	5’ATAAAGAGGCAGCGGAAAGC3’	105 bp
vgrG2rev	5’TCAGCAGTGAACTGGTGCTC3’	
clpV2for	5’AACTGACCGATTTGCGTGAT3’	108 bp
clpV2rev	5’CTTCCTGACGCAACGTCATA3’	
tus for	5’CGATAACCTTTCGCAAGCAGCGTT3’	138 bp
tusrev	5’GGCAAATGACGATGCACCCATTCA3’	

For cloning

Name of sequence	Sequence
Clpv1 F EcoRI	CACGGAATTCAACATACAGGGTGGGCGTTA
Clpv1 R XbaII	GCAGTCTAGA TGTAACAGCACATGGGCAAC
Clpv1 Fcheckfus	GGCGGCGAGAAAGTGTTTAT
Clpv2 F EcoRI	CACGGAATTC GCATTGAGTTCCGCTACGAG
Clpv2 R XbaII	GCAGTCTAGA GCACAGTATGGGTTAAGGCG
Clpv2 Fcheckfus	AAGCTGGTCAGAGTAGCTCC
dotU1 F EcoRI	CACGGAATTC CAGGACGTGATAAAAGCGGG
dotU1 R XbaII	GCAGTCTAGA GGCCTGGACATCGGTTACTA

For cloning

Name of sequence	Sequence
dotU1 Fcheckfus	CATCCAGCAGTCGAAAGTGG
dotU2 F EcoRI	CACGGAATTC ATCTGTCCGTTCGTTCCTCA
dotU2 R XbaII	GCAGTCTAGA GTTCCACCACCGTCAGAAAC
dotU2 Fcheckfus	GTTCTCCCTGCTCAGCGA
icmF1 F EcoRI	CACGGAATTC CCAGTTTCACCGCCAGTTTT
icmF1 R XbaII	GCAGTCTAGA AAAATCCATGCCAGCGTCAG
icmF1 Fcheckfus	TTGTTTAACGACCGCACTCC
icmF2 F EcoRI	CACGGAATTC GCATGTCTGGGTCTCTGGTT
icmF2 R XbaII	GCAGTCTAGA CCAGAAACCGTAACGCTGAG
icmF2 Fcheckfus	ATCGTCATTTGTACGGCAGC
vgrG1 F EcoRI	CACGGAATTC AAGGTTGAGTGGGAGCACAT
vgrG1 R XbaII	GCAGTCTAGA CGTCAACCTCCAGCGTAAAG

For cloning

Name of sequence	Sequence
vgrG1 Fcheckfus	GCGACGATTGTGGACATGAA
vgrG2 F EcoRI	CACGGAATTC ACAGCCTGTATGACCAGCTT
vgrG2 R XbaII	GCAGTCTAGA GCTGTTCGTGGCCTGTAAAA
vgrG2 Fcheckfus	CGCAACACCGTCATTCTGAT
pVIK 112 on	GTTGCACCACAGATGAAACG

- Underlined are restriction cutting sites

Supplementary Table 2. Characterization of 187 avian pathogenic *E. coli* isolates in terms of the presence of *clpV, vgrG, icmF,* and *dotU* genes and PS.

	Type 6 Secretion System 1				
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS[1]
RS001	0	0	0	1	7,93
RS002	0	0	0	1	1
RS003	0	0	1	1	10
RS004	0	0	0	0	7,07
RS005	0	0	0	0	9,15
RS007	0	0	0	1	10
RS008	0	0	0	1	10
RS009	0	0	0	1	10
RS010	0	0	0	1	9,29
RS011	0	0	0	1	10
RS013	0	0	0	0	1
RS017	0	0	1	1	9,65
RS018	0	0	1	1	0
RS020	0	0	1	1	5,93
RS021	0	0	1	1	1
RS022	0	0	1	1	1
RS023	0	0	0	0	6,43
RS024	0	0	1	1	1
RS025	0	0	1	1	1
RS026	0	0	1	1	0
RS027	0	0	1	1	0
RS028	0	0	1	1	10
RS029	0	0	1	1	7,43
RS030	0	0	0	1	6,29
RS031	0	0	1	1	10
RS032	0	0	1	1	1
RS033	0	0	0	1	8,79
RS037	0	0	1	1	10
RS038	0	0	0	1	7,85
RS039	0	0	0	1	1
RS040	0	0	1	1	8,57
RS042	0	0	1	1	6,71
RS043	0	0	1	1	0,5
RS044	0	0	1	1	7,57
RS045	0	0	0	0	10
RS046	0	0	1	1	0
RS047	0	0	0	1	4,5

Type 6 Secretion System 1					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
RS049	0	0	0	1	8,93
RS050	0	0	1	1	7,71
RS071	0	0	1	1	7,07
RS084	0	0	0	0	6,42
RS085	0	0	0	1	8,65
RS086	0	0	0	1	10
RS087	0	0	0	1	9,15
RS096	0	0	0	1	5,43
RS097	0	0	0	1	7,43
RS106	0	0	1	1	8,29
RS107	0	0	1	1	1
RS108	0	0	1	1	9,29
RS111	0	0	0	1	3,14
PR2	0	0	1	1	10
PR3	0	0	0	1	6,29
PR4	0	0	1	1	10
PR6	0	0	0	1	7,29
PR7	0	0	1	1	7,57
PR8	1	1	1	1	2,5
PR9	1	1	1	1	6,29
PR10	0	0	1	1	9,15
PR11	0	0	1	1	10
PR12	0	0	0	1	6,93
PR13	1	1	1	1	10
PR14	1	1	0	1	3,85
PR15	0	0	1	1	5,57
PR16	1	1	1	1	7,57
PR17	1	1	1	1	9,29
PR18	1	1	0	1	7,29
PR19	0	0	0	1	7,93
PR20	0	0	0	0	7,79
PR21	0	0	1	1	9,29
PR22	1	1	1	1	4,71
PR23	1	1	1	1	8,57
PR24	1	1	1	1	2,5
PR25	1	1	1	1	8,65
PR26	1	1	1	1	5,78
PR27	0	0	1	1	10
PR28	0	0	1	1	10
PR29	1	1	1	0	10
PR30	0	0	1	1	10

Type 6 Secretion System 1					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
PR31	0	0	1	1	10
PR32	0	0	0	0	6,93
PR33	0	0	1	1	7,07
PR34	1	1	0	0	10
PR35	1	1	1	0	10
PR41	1	1	1	0	10
PR42	1	1	1	1	7,29
PR43	1	1	1	1	10
PR44	0	0	0	1	10
PR45	0	0	1	1	8,65
PR46	1	1	1	1	10
PR47	0	0	0	1	9,65
PR48	0	1	1	1	5,15
PR49	1	1	0	1	7,29
PR50	1	1	0	1	10
PR51	0	0	1	1	7,29
PR52	0	0	1	1	6,29
PR53	0	0	1	1	10
PR54	0	0	1	1	6,43
PR55	0	0	1	1	10
PR56	1	1	0	0	6,29
PR57	1	1	0	0	10
PR58	0	0	0	0	5,57
PR59	0	0	0	0	10
PR61	0	0	0	1	10
PR62	0	0	1	1	10
PR64	0	0	0	0	9,65
PR65	0	0	0	1	10
PR68	1	1	1	1	10
PR69	1	1	0	1	6,29
PR70	0	0	0	1	10
PR71	0	0	0	0	9,29
PR72	0	0	0	0	10
PR73	0	0	0	1	9,65
PR74	0	0	1	1	8,07
PR75	0	0	0	1	10
PR76	0	0	1	1	7,29
PR77	0	0	0	1	6,29
PR78	0	0	0	1	10
PR79	0	0	0	1	10
PR80	0	0	0	0	8,29

Type 6 Secretion system 1					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
PR81	0	0	0	1	10
PR82	0	0	0	1	9,65
PR83	0	0	1	1	10
PR84	0	0	0	0	5,93
PR89	0	0	0	1	5,93
PR91	0	0	0	1	7,57
PR92	0	0	0	1	7,85
PR93	0	0	0	1	9,65
PR94	0	0	0	0	8,65
PR95	0	0	0	1	10
PR96	0	0	0	1	6,29
PR97	0	0	0	0	6,22
PR98	0	0	0	1	6,36
PR99	0	0	1	1	10
PR100	0	0	1	1	5,93
PR101	0	0	0	1	6,36
PR102	0	0	1	1	10
PR103	0	0	1	1	6,43
PR104	0	0	0	1	10
PR105	0	0	1	1	6,22
PR106	0	0	0	1	10
PR107	0	0	0	0	6,29
PR108	0	0	0	1	10
PR109	0	0	1	1	5,93
PR110	0	0	1	1	6,93
PR111	0	0	0	1	6,57
PR112	0	0	0	1	4,93
PR113	0	0	0	1	6,07
PR114	0	0	0	1	9,65
PR115	0	0	1	1	10
PR116	0	0	0	0	8,29
PR117	0	0	1	1	10
PR118	0	0	0	1	4,71
PR119	0	0	0	1	6,79
PR120	0	0	0	0	2
PR121	0	0	0	0	5,57
PR122	0	0	1	1	2,5
PR123	0	0	0	1	10
PR124	0	0	1	1	5,57
PR125	0	0	0	1	6,29
PR126	0	0	0	0	5,71

Type 6 Secretion System 1					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
PR127	0	0	0	0	6,93
PR129	0	0	0	0	1
PR130	0	0	1	1	6,43
PR133	0	0	0	1	7,43
PR135	0	0	0	0	10
PR136	0	0	1	1	8,79
PR137	0	0	0	0	6,79
PR138	0	0	0	0	7,43
PR139	0	0	0	1	10
PR140	0	0	0	0	6,93
PR142	0	0	0	1	10
PR143	0	0	0	0	10
PR144	0	0	0	0	6,93
PR145	0	0	0	0	10
PR146	0	0	0	1	8,65
PR147	0	0	0	0	6,79
PR148	0	0	0	1	10
Type 6 Secretion System 2					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
RS001	0	0	0	0	7,93
RS002	0	0	0	0	1
RS003	0	0	0	0	10
RS004	0	0	1	1	7,07
RS005	0	0	1	1	9,15
RS007	0	0	0	0	10
RS008	0	0	0	0	10
RS009	0	0	0	0	10
RS010	0	0	0	0	9,29
RS011	0	0	0	0	10
RS013	1	1	1	1	1
RS017	0	1	1	1	9,65
RS018	1	1	1	1	0
RS020	0	0	1	1	5,93
RS021	0	0	0	0	1
RS022	1	1	1	1	1
RS023	0	0	0	0	6,43
RS024	0	0	0	0	1
RS025	1	1	1	1	1
RS026	0	0	0	0	0
RS027	0	0	0	0	0
RS028	0	0	0	0	10

Type 6 Secretion System 2					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
RS029	0	0	0	0	7,43
RS030	0	0	0	0	6,29
RS031	0	0	0	0	10
RS032	0	0	0	0	1
RS039	0	0	0	0	1
RS040	0	0	0	0	8,57
RS042	0	0	0	0	6,71
RS043	0	0	0	1	0,5
RS044	0	0	1	1	7,57
RS045	1	0	0	0	10
RS046	0	0	1	0	0
RS047	1	1	1	1	4,5
RS049	1	1	1	1	8,93
RS050	0	1	1	1	7,71
RS071	0	0	0	0	7,07
RS084	1	1	1	1	6,42
RS085	0	0	0	0	8,65
RS086	0	0	0	0	10
RS087	0	0	0	0	9,15
RS096	1	1	1	1	5,43
RS097	0	0	0	0	7,43
RS106	0	0	0	0	8,29
RS107	0	0	0	0	1
RS108	0	0	0	0	9,29
RS111	0	0	0	0	3,14
PR2	0	0	0	0	10
PR3	0	0	0	0	6,29
PR4	0	0	0	0	10
PR6	0	0	0	0	7,29
PR7	0	0	0	0	7,57
PR8	0	0	0	0	2,5
PR9	0	0	0	0	6,29
PR10	1	0	1	1	9,15
PR11	0	0	1	1	10
PR12	0	0	0	0	6,93
PR13	1	1	1	1	10
PR14	1	0	1	1	3,85
PR15	0	0	1	0	5,57
PR16	1	1	1	1	7,57
PR17	1	1	1	1	9,29
PR18	0	0	0	0	7,29

Type 6 Secretion System 2					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
PR19	*0*	*0*	*0*	*0*	7,93
PR20	*0*	*0*	*1*	*1*	7,79
PR21	*0*	*0*	*0*	*0*	9,29
PR22	*1*	*1*	*1*	*1*	4,71
PR23	1	1	1	1	8,57
PR24	0	1	1	1	2,5
PR25	0	1	1	1	8,65
PR26	1	1	1	1	5,78
PR27	0	0	0	0	10
PR28	0	0	0	0	10
PR29	1	1	1	1	10
PR30	0	0	0	0	10
PR31	0	0	0	0	10
PR32	1	1	1	1	6,93
PR33	1	0	1	1	7,07
PR34	0	1	1	1	10
PR35	0	1	1	1	10
PR36	0	1	1	1	10
PR38	0	0	1	1	6,29
PR39	0	1	1	1	10
PR40	0	1	1	1	10
PR41	0	1	1	1	10
PR42	0	1	1	1	7,29
PR43	0	0	0	0	10
PR44	0	0	0	0	10
PR45	0	0	0	0	8,65
PR46	1	1	1	1	10
PR47	0	0	0	0	9,65
PR48	0	0	1	1	5,15
PR49	1	1	1	1	7,29
PR50	1	1	1	1	10
PR51	0	0	1	1	7,29
PR52	0	0	0	0	6,29
PR53	0	0	0	0	10
PR54	0	0	0	0	6,43
PR55	0	0	0	0	10
PR56	0	0	1	1	6,29
PR57	0	0	1	1	10
PR58	1	1	1	1	5,57
PR59	0	0	1	1	10
PR61	0	0	0	0	10

Type 6 Secretion System 2					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
PR62	0	0	0	0	10
PR64	0	1	1	1	9,65
PR65	0	0	0	0	10
PR68	0	0	0	0	10
PR69	0	0	1	1	6,29
PR70	1	1	1	1	10
PR71	1	1	1	1	9,29
PR78	0	0	0	0	10
PR79	0	0	0	0	10
PR80	1	1	1	1	8,29
PR81	0	0	1	1	10
PR82	0	0	1	1	9,65
PR83	0	0	0	0	10
PR84	1	1	1	1	5,93
PR85	1	1	1	1	10
PR86	0	0	0	0	8,21
PR87	1	1	1	1	5,35
PR88	1	1	1	1	10
PR89	1	1	1	1	5,93
PR91	0	0	0	0	7,57
PR92	1	1	1	1	7,85
PR93	1	1	1	1	9,65
PR94	1	1	1	1	8,65
PR95	0	1	1	1	10
PR96	1	1	1	1	6,29
PR97	1	1	1	1	6,22
PR98	0	0	0	0	6,36
PR99	1	1	1	1	10
PR100	1	1	1	1	5,93
PR101	1	1	1	1	6,36
PR102	0	1	1	1	10
PR103	1	1	1	1	6,43
PR104	0	0	1	1	10
PR105	0	0	0	0	6,22
PR106	1	1	1	1	10
PR107	1	1	1	1	6,29
PR108	1	1	1	1	10
PR109	1	1	1	1	5,93
PR110	0	0	1	1	6,93
PR111	1	0	1	1	6,57
PR112	1	1	1	1	4,93

Type 6 Secretion System 2					
Strain	*icmF*	*dotU*	*clpV*	*vgrG*	PS
PR113	1	1	1	1	6,07
PR114	0	0	0	0	9,65
PR115	0	0	0	0	10
PR116	0	1	1	1	8,29
PR120	1	1	1	1	2
PR121	1	1	1	1	5,57
PR122	1	1	1	1	2,5
PR123	0	0	1	1	10
PR124	0	1	1	1	5,57
PR125	0	1	1	1	6,29
PR126	1	1	1	1	5,71
PR127	1	1	1	1	6,93
PR129	1	1	1	1	1
PR130	0	0	0	0	6,43
PR131	1	1	1	1	3,85
PR132	0	0	0	0	10
PR133	1	1	1	1	7,43
PR135	0	0	1	1	10
PR136	0	0	0	0	8,79
PR137	1	1	1	1	6,79
PR138	0	0	1	1	7,43
PR139	0	0	0	0	10
PR140	1	1	1	1	6,93
PR142	0	0	0	0	10
PR143	1	1	1	1	10
PR144	1	1	1	1	6,93
PR145	1	1	1	1	10
PR146	1	1	1	1	8,65
PR147	1	1	1	1	6,79
PR148	0	0	0	0	10

1. The PS values of the APEC strains (PR and RS) were adopted from previous publications (Barbieri*et al*., 2013; Barbieri*et al*., 2014).

6. CONCLUSÕES E PERSPECTIVAS

Neste trabalho, foi realizada a triagem dos genes de virulência plasmidiais *iroN*, *ompT*, *hlyF*, *iss* e *iutA,* previamente estabelecidos como preditores da virulência de APEC, em uma coleção de isolados de *E. coli* patogênicas aviárias (APEC), de cama de aviário (A_{litter}) e da cloaca de aves sadias (A_{fecal}), no intuito de verificar a prevalência desses genes nas diferentes classes de isolados bem como sua relação com a virulência de APEC. Entre as cepas APEC, 71% apresentaram todos os genes analisados, confirmando uma alta prevalência destes genes entre cepas patogênicas. Além disso, observou-se que cepas com dois ou mais dos genes analisados tem maior probabilidade de serem patogênicas do que aquelas que apresentaram um ou nenhum dos genes. No entanto, 31% de cepas não patogênicas apresentaram todos os genes analisados, ou seja, uma grande proporção de cepas não-patogênicas seria classificada como patogênicas, caso o critério da presença dos genes analisados fosse utilizada para inferir a patogenicidade dessas cepas. Dessa forma, a PCR pentaplex definida como um método de diagnóstico rápido da patogenicidade de cepas APEC não demonstrou reprodutibilidade quando aplicada a uma coleção de isolados brasileiros. Embora esses resultados não estejam de acordo com o esperado, a realização desse estudo foi importante por se tratar da caracterização de um grande número de amostras em relação à filogenia, à prevalência de genes associados a plasmídios do tipo ColV e a virulência *in vivo*.

Na segunda parte do trabalho, foi realizada a análise da frequência dos genes *icmF*, *dotU*, *clpV* e *vgrG*, componentes do T6SS, em 187 isolados APEC. O T6SS tem sido associado a diferentes interações bacterianas, e mais recentemente, tem sido associado a cepas de *Escherichia coli* patogênica aviária. Em algumas cepas bacterianas positivas para os genes testados, analisou-se a capacidade de resistir à predação por *D. discoideum*, fenótipo associado à funcionalidade do T6SS. Observou-se que algumas cepas que apresentam ao menos o uma cópia do gene *vgrG*, que codifica uma proteína estrutural e efetora do sistema, apresentam resistência a predação *D. discoideum*, o que pode indicar

a presença de um T6SS funcional. Além disso, a expressão do efetor *vgrG* e da ATPase *clpV* foi analisada em três cepas da coleção, selecionadas de acordo com a demonstração ou não do fenótipo e com o Índice de Patogenicidade previamente atribuído a essas cepas. Como previamente discutido, não foi observada diferença significativa na expressão dos genes analisados pelas diferentes cepas. Analisou-se, então, a expressão dos mesmos genes pela cepa APEC submetida a contato com células eucarióticas, com o objetivo de verificar se a expressão desses genes seria induzida pelo contato com células eucarióticas, como demonstrado em outras cepas APEC bem como em outras espécies bacterianas. Ao contrário do esperado, não houve indução da expressão pelo contato com células eucarióticas. Por último, foi analisada a secreção de proteínas pela cepa APEC O1 no meio de cultura ou durante o contato com células eucarióticas. Foram observados diferentes padrões de secreção protéica quando comparadas as duas condições. Uma análise futura das proteínas secretadas nas duas condições ajudará a elucidar o envolvimento do T6SS na interação entre a cepa APEC O1 e células eucarióticas. Ainda em andamento, está a construção de cepas transformantes, com a perspectiva de testar a expressão dos genes do sistema sob diversas condições, de uma forma mais prática e rápida se comparada ao qRT-PCR. Em relação ao T6SS, este trabalho apresenta a primeira análise epidemiológica de uma coleção de cepas APEC em relação à presença de genes componentes do T6SS.

REFERÊNCIAS GERAIS

Abdallah, A. M.; Gey Van Pittius, N. C.; Champion, P. A.; Cox, J.; Luirink, J.; Vandenbroucke-Grauls, C. M.; Appelmelk, B. J.; Bitter, W. (2007) Type VII secretion: mycobacteria show the way. Nature Reviews in Microbiology., v. 5, n. (11), p. 883-91

Barbieri, N. L.; De Oliveira, A. L.; Tejkowski, T. M.; Pavanelo, D. B.; Rocha, D. A.; Matter, L. B.; Callegari-Jacques, S. M.; De Brito, B. G.; Horn, F. (2013) Genotypes and pathogenicity of cellulitis isolates reveal traits that modulate APEC virulence. Plos One, v. 8, n. 8, p. e72322

Barbieri, N. L.; Oliveira, A. L.; Tejkowski, T. M.; Pavanelo, D. B.; Matter, L. B.; Pinheiro, S. R. S.; Vaz, T. M. I.; Brito, B. G.; Nolan, L. K.; Logue, C. M.; Horn, F. (2014) Molecular characterization and clonal relationships among *Escherichia coli* strains isolated from broiler chickens with colisepticemia. Foodborne Pathogens and Disease, v. accepted for publication,

Barbieri, N. L.; Tejkowski, T. M.; Oliveira, A. L.; Brito, B. G.; Horn, F. (2012) Characterization of extra-intestinal *Escherichia coli* isolated from a peacock (*Pavo cristatus*) with colisepticemia. Avian Diseases, v. *56*, n. 2, p. 436-440

Barnes, H. J.; Gross, W. B. Colibacilosis. In: CALNEK, B. W. (Ed.). (1997) Disease of Poultry. Ames: Iowa state university press, v.10, 1997. p.131-141.

Bingle, L. E. H.; Bailey, C. M.; Pallen, M. J. (2008) Type VI secretion: a beginner's guide. Current Opinion in Microbiology, v. 11, n. 1, p. 3-8

Boyer, F.; Fichant, G.; Berthod, J.; Vandenbrouck, Y.; Attree, U. (2009) Dissecting the bacterial type VI secretion system by a genome wide in silico analysis: what can be

learned from available microbial genomic resources? BMC Genomics, v. 10, n. 104,

Cascales, E. (2008) The type VI secretion toolkit. Embo Reports, v. 9, n. 8, p. 735-741

Tenth Edition M02-A10. 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA: Clinical and Laboratory Standards Institute

De Pace, F.; Boldrin De Paiva, J.; Nakazato, G.; Lancellotti, M.; Sircili, M. P.; Guedes Stehling, E.; Dias Da Silveira, W.; Sperandio, V. (2011) Characterization of IcmF of the type VI secretion system in an avian pathogenic *Escherichia coli* (APEC) strain. Microbiology, v. 157, n. Pt 10, p. 2954-62

De Pace, F.; Nakazato, G.; Pacheco, A.; De Paiva, J. B.; Sperandio, V.; Da Silveira, W. D. (2010) The type VI secretion system plays a role in type 1 fimbria expression and pathogenesis of an avian pathogenic *Escherichia coli* strain. Infection and Immunity, v. 78, n. 12, p. 4990-4998

Decoin, V.; Barbey, C.; Bergeau, D.; Latour, X.; Feuilloley, M. G. J.; Orange, N.; Merieau, A. (2014) A Type VI Secretion System Is Involved in *Pseudomonas fluorescens* Bacterial Competition. PLoS ONE, v. 9, n. 2, p. e89411

Desvaux, M.; Hébraud, M.; Talon, R.; Henderson, I. R. (2009) Secretion and subcellular localizations of bacterial proteins: a semantic awareness issue. Trends in Microbiology, v. 17, n. 4, p. 139-45

Dho-Moulin, M.; Fairbrother, J. M. (1999) Avian pathogenic *Escherichia coli* (APEC). Veterinary Research, v. 30, n. 2-3, p. 299-316

Dobrindt, U. (2005) (Patho-)genomics of *Escherichia coli*. Int J Med Microbiol, v. 295, n. (6-7), p. 357-71

Dziva, F.; Stevens, M. P. (2008) Colibacillosis in poultry: unravelling the molecular basis of virulence of avian pathogenic *Escherichia coli* in their natural hosts. Avian Pathology, v. 37, n. 4, p. 355-366

Falkow, S. (1991) BACTERIAL ENTRY INTO EUKARYOTIC CELLS. Cell, v. 65, n. 7, p. 1099-1102

Ferreira, A. J.; Knöbl, T. Colibacilose Aviária. In: BERCHIERI, A. e MACARI, M. (Ed.). (2000) Doença das Aves. Campinas: FACTA, v.197-205, 2000.

Filloux, A.; Hachani, A.; Bleves, S. (2008) The bacterial type VI secretion machine: yet another player for protein transport across membranes. Microbiology-Sgm, v. 154, p. 1570-1583

Ginns, C. A.; Benham, M. L.; Adams, L. M.; Whithear, K. G.; Bettelheim, K. A.; Crabb, B. S.; Browning, G. F. (2000) Colonization of the respiratory tract by a virulent strain of avian *Escherichia coli* requires carriage of a conjugative plasmid. Infection and Immunity, v. 68, n. 3, p. 1535-1541

Johnson, T. J.; Giddings, C. W.; Horne, S. M.; Gibbs, P. S.; Wooley, R. E.; Skyberg, J.; Olah, P.; Kercher, R.; Sherwood, J. S.; Foley, S. L.; Nolan, L. K. (2002) Location of increased serum survival gene and selected virulence traits on a conjugative R plasmid in an avian *Escherichia coli* isolate. Avian Diseases, v. 46, n. 2, p. 342-352

Johnson, T. J.; Siek, K. E.; Johnson, S. J.; Nolan, L. K. (2006) DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian *Escherichia coli* strains. Journal of Bacteriology, v. 188, n. 2, p. 745-758

Johnson, T. J.; Wannemuehler, Y.; Doetkott, C.; Johnson, S. J.; Rosenberger, S. C.; Nolan, L. K. (2008) Identification of minimal predictors of avian pathogenic

Escherichia coli virulence for use as a rapid diagnostic tool. Journal of Clinical Microbiology, v. 46, n. 12, p. 3987-3996

Kaper, J. B.; Nataro, J. P.; Mobley, H. L. T. (2004) Pathogenic *Escherichia coli*. Nature Reviews Microbiology, v. 2, n. 2, p. 123-140

Kostakioti, M.; Newman, C. L.; Thanassi, D. G.; Stathopoulos, C. (2005) Mechanisms of protein export across the bacterial outer membrane. Journal of Bacteriology, v. 187, n. (13), p. 4306-14

Ma, J.; Bao, Y.; Sun, M.; Dong, W.; Pan, Z.; Zhang, W.; Lu, C.; Yao, H. (2014) Two functional type VI secretion systems in avian pathogenic *Escherichia coli* are involved in different pathogenic pathways. Infect Immun, v. 82, n. 9, p. 3867-79

Ma, J.; Sun, M.; Bao, Y.; Pan, Z.; Zhang, W.; Lu, C.; Yao, H. (2013) Genetic diversity and features analysis of type VI secretion systems loci in avian pathogenic *Escherichia coli* by wide genomic scanning. Infect Genet Evol, v. 20, p. 454-64

Macintyre, D. L.; Miyata, S. T.; Kitaoka, M.; Pukatzki, S. (2010) The *Vibrio cholerae* type VI secretion system displays antimicrobial properties. Proceedings of the National Academy of Sciences of the United States of America, v. 107, n. 45, p. 19520–19524

Mougous, J. D.; Cuff, M. E.; Raunser, S.; Shen, A.; Zhou, M.; Gifford, C. A.; Goodman, A. L.; Joachimiak, G.; Ordoñez , C. L.; Lory, S.; Walz, T.; Joachimiak, A. *et al.* (2006) A virulence locus of *Pseudomonas aeruginosa* encodes a protein secretion apparatus. Science, v. 312, n. 5779, p. 1526-30.

Ngeleka, M.; Kwaga, J. K. P.; White, D. G.; Whittam, T. S.; Riddell, C.; Goodhope, R.; Potter, A. A.; Allan, B. (1996) *Escherichia coli* cellulitis in broiler chickens: Clonal

relationships among strains and analysis of virulence-associated factors of isolates from diseased birds. Infection and Immunity, v. 64, n. 8, p. 3118-3126

Pukatzki, S.; Kessin, R. H.; Mekalanos, J. J. (2002) The human pathogen *Pseudomonas aeruginosa* utilizes conserved virulence pathways to infect the social amoeba *Dictyostelium discoideum*. Proceedings of the National Academy of Sciences of the United States of America, v. 99, n. 5, p. 3159-64

Pukatzki, S.; Ma, A. T.; Revel, A. T.; Sturtevant, D.; Mekalanos, J. J. (2007) Type VI secretion system translocates a phage tail spike-like protein into target cells where it cross-links actin. Proceedings of the National Academy of Sciences of the United States of America, v. 104, n. 39, p. 15508-13

Pukatzki, S.; Ma, A. T.; Sturtevant, D.; Krastins, B.; Sarracino, D.; Nelson, W. C.; Heidelberg, J. F.; Mekalanos, J. J. (2006) Identification of a conserved bacterial protein secretion system in *Vibrio cholerae* using the *Dictyostelium* host model system. Proceedings of the National Academy of Sciences of the United States of America, v. 103, n. 5, p. 1528-33

Pukatzki, S.; Mcauley, S. B.; Miyata, S. T. (2009) The type VI secretion system: translocation of effectors and effector-domains. Curr Opin Microbiol, v. 12, n. 1, p. 11-7

Reis, R. S.; Horn, F. (2010) Enteropathogenic *Escherichia coli, Samonella, Shigella* and *Yersinia*: cellular aspects of host-bacteria interactions in enteric diseases. Gut Pathogens, v. 2, n. 2, p. 8

Rodriguez-Siek, K. E.; Giddings, C. W.; Doetkott, C.; Johnson, T. J.; Nolan, L. K. (2005) Characterizing the APEC pathotype. Veterinary Research, v. 36, n. 2, p. 241-256

Russo, T. A.; Johnson, J. R. (2000) Proposal for a new inclusive designation for extraintestinal pathogenic isolates of *Escherichia coli*: ExPEC. Journal of Infectious Diseases, v. 181, n. 5, p. 1753-1754

Schell, M. A.; Ulrich, R. L.; Ribot, W. J.; Brueggemann, E. E.; Hines, H. B.; Chen, D.; Lipscomb, L.; Kim, H. S.; Mrázek, J.; Nierman, W. C.; Deshazer, D. (2007) Type VI secretion is a major virulence determinant in *Burkholderia mallei*. Molecular Microbiology, v. 64 n. 6, p. 1466-85

Segal, G.; Purcell, M.; Shuman, H. A. (1998) Host cell killing and bacterial conjugation require overlapping sets of genes within a 22-kb region of the *Legionella pneumophila* genome. Proceedings of the National Academy of Sciences of the United States of America, v. 95, n. 4, p. 1669-74

Shrivastava, S.; Mande, S. S. (2008) Identification and Functional Characterization of Gene Components of Type VI Secretion System in Bacterial Genomes. Plos One, v. 3, n. 8, p. e2955

Tseng, T.-T.; Tyler, B. M.; Setubal, J. C. (2009) Protein secretion systems in bacterial-host associations, and their description in the Gene Ontology. BMC Microbiology, v. 9,

Ubabef. União Brasileira de Avicultura, Relatório Anual 2014. www.ubabef.com.br. 2014

Vogel, J. P.; Andrews, H. L.; Wong, S. K.; Isberg, R. R. (1998) Conjugative transfer by the virulence system of *Legionella pneumophila*. Science, v. 279, n. 5352, p. 873-6

Wang, S.; Dai, J.; Meng, Q.; Han, X.; Han, Y.; Zhao, Y.; Yang, D.; Ding, C.; Yu, S. (2014) DotU expression is highly induced during in vivo infection and responsible

for virulence and Hcp1 secretion in avian pathogenic *Escherichia coli*. Front Microbiol, v. 5, p. 588

Waters, V. L.; Crosa, J. H. (1991) Colicin V virulence plasmids. Microbiol Rev, v. 55, n. 3, p. 437-50

Zheng, J.; Leung, K. Y. (2007) Dissection of a type VI secretion system in *Edwardsiella tarda*. Molecular Microbiology, v. 66, n. 5, p. 1192-206.

Zoued, A.; Brunet, Y. R.; Durand, E.; Aschtgen, M. S.; Logger, L.; Douzi, B.; Journet, L.; Cambillau, C.; Cascales, E. (2014) Architecture and assembly of the Type VI secretion system. Biochim Biophys Acta, v. 1843, n. 8, p. 1664-73