Evaluating Virulence Factors as Targets for Antivirulence Veterinary Drugs for Avian Pathogenic *Escherichia coli* to Combat Colibacillosis in Chickens

[1,2] Kachrani, Hershil, [1]Harbottle, Heather, [1]Martinez, Marilyn, [3]Foley, Steve, [1]Gilbert, Jeff

[1]FDA/CVM, Office of New Animal Drug Evaluation, Rockville, MD

[2] University of New England, College of Pharmacy, Portland, ME

[3] FDA/NCTR, Jefferson, AR

Abstract

Background: Colibacillosis, caused by avian pathogenic *Escherichia coli* (APEC), was selected as a model infection for exploring the potential utility of, and potential regulatory approaches for, evaluating antivirulence drugs for the treatment of infectious diseases**.** Colibacillosis is an infectious disease affecting poultry flocks causing acute fatal septicemia, subacute pericarditis, airsacculitis, and other sequelae. Although production strategies have been adopted to control the spread of this disease, treatment continues to rely on antimicrobial drug therapy, including tetracyclines, cephalosporins, and aminoglycosides.
Purpose: Using colibacillosis as a model infection, this project explores the kinds of information and data necessary to support CVM's pre-approval evaluations of these alternatives to traditional antimicrobial drugs. This includes the use of genetic assessments to confirm the involvement of specific virulence mechanisms in a given disease process, and their potential to promote co-selection of antimicrobial resistance.
Methodology: Existing scientific literature and genomic data are being mined to address CVM's concerns regarding unintended consequences, such as co-selection of antimicrobial resistance. A review of published literature was conducted to choose virulence genes as proof-of-concept drug targets. Three genes were chosen, representing adhesins (type I fimbrae, *fimH*), iron acquisition (aerobactin operon, *iutA*), and toxins (colicin, *cvaC*). A total of 66 APEC isolates from chicken sources were selected for inclusion from the National Antimicrobial Resistance Monitoring System (NARMS) (25 retail chicken breast isolates, 26 chicken cecal isolates), 12 isolates from chickens with colibacillosis (Mageiros et al. 2021), 2 positive controls from chickens with colibacillosis from the literature, and a negative control extra-intestinal pathogenic *E. coli* (ExPEC). Isolates were identified as APEC by detection of *iss* and *tsh* genes from chicken sources. Whole genome sequences and metadata were obtained from the National Center for Biotechnology Information (NCBI). Antimicrobial resistance gene, virulence gene, and plasmid detection data were obtained in addition to antimicrobial susceptibility testing (AST) data, where available. Maximum likelihood trees based on single nucleotide polymorphisms (SNPs) or amino acid changes were created by Dr. Chih-Hao Hsu for each of the three virulence genes (*cvaC, iutA, fimH*) in each isolate. Correlations between virulence gene detection, antimicrobial resistance genotype or phenotype, and type of source were assessed.
Conclusion: Preliminary results show that 100% of retail and cecal isolates were positive for *iutA*, and 100% of cecal and diseased animal isolates were positive for *fimH;* however, positive control isolates were not positive for all three target genes. This suggests that virulence genes are being propagated among the *E. coli* residing in poultry. The *cvaC* gene was detected least frequently of the three gene candidates (76% retail and cecal isolates, and 25% in diseased animals). The latter was a surprising finding, raising the need for further study. SNPs were not detected in *cvaC* but did cause amino acid changes in *iutA* and more commonly in *fimH*. The IncFIB plasmid was identified in 94% of retail and cecal isolates, and 83% of diseased animal isolates. The percent of isolates exhibiting antimicrobial resistance was low in all sources. This study will elucidate the kind of information and data needed for development of a pre-approval, regulatory science "roadmap" to evaluate antivirulence drugs across a wide scope of diseases impacting animal and human health.

Introduction

The use of antimicrobial drugs in food-producing animals has come under recent scrutiny due to consumer concerns and recent action by the FDA limiting the use of medically important antimicrobials to judicious uses only, removing long-standing production indications from their approved use status. With the reduction in availability of antimicrobials for use in food-producing animals, and a move towards the marketing of "no antibiotics ever" or organic food products, interest in the use of alternative molecules, such as drugs that target virulence factors, to treat food-animal diseases has increased. To properly evaluate drugs that target virulence factors, a proof-of-concept study using colibacillosis in chickens, caused by APEC, was initiated by CVM. Colibacillosis was selected as the infectious process because of the wealth of available genomic data; moreover, because *E. coli* can be pathogenic both in humans and veterinary species, this work carries important One-Health implications. Studying a pathogen's genome provides insights into the disease-producing process, bacterial mechanisms to resist antimicrobial drug actions, and potential compensatory survival mechanisms, including gene transfer. Data to investigate this impact will be investigated using whole genome sequencing and phenotypic resistance data where available from publicly available information in the literature, from NCBI, and the NARMS program. Co-selection of antimicrobial resistance on the chromosome or on mobile genetic elements, such as plasmids, will be investigated. Because of the novelty of this alternative approach, CVM faces a number of critical questions that have yet to be addressed including: 1) are there prominent virulence factors that can provide effective targets for treatment in the majority of disease outbreaks, or are there strain variations that could render antivirulence therapies outbreak-specific? 2) would genetic testing be needed to identify when a specific antivirulence drug may be appropriate? 3) could use of these treatments increase the potential for selection of antimicrobial resistance? 4) what are the risks for plasmid transfer between strains, and are there associated risks from a human food safety perspective, including transmission of APEC to humans *via* food? 5) what are the risks for inducing foodborne disease in humans, and is there a need to include virulence factors as part of our human food safety assessment (Martinez et al. 2019)? This project will explore these questions from the perspective of a single virulence factor, including type 1 fimbriae (*fimH*), aerobactin operon for iron-acquisition (*iutA*) and colicin (bacterial competition factor, *cvaC*), that would have the greatest impact on poultry morbidity and mortality. CVM will utilize this information and data to develop guidelines for industry for regulatory evaluation of antivirulence drugs for food animals.

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Materials and Methods

Virulence gene selection: A comprehensive review of the literature was conducted in order to choose virulence genes as proof-of-concept drug targets (Table 1). Three genes were chosen which vary in function: Adhesion - type I fimbrae (*fimH*), Iron acquisition – aerobactin operon (*iutA*), and toxins – colicin (*cvaC*).
Isolate selection: A total of 66 APEC isolates from chicken sources were selected for this project from NARMS (25 retail chicken breast isolates from FDA/CVM, 26 chicken cecal isolates from USDA/FSIS), 12 isolates from chickens with colibacillosis from published literature (Mageiros et al. 2021), two positive controls from chickens with colibacillosis (Johnson et al. 2007, Mangiamele et al. 2013), and a negative control extra-intestinal pathogenic *E. coli* (ExPEC) from retail ground beef from the NARMS program. Isolates were identified as APEC by the detection of the *iss* and *tsh* genes from chicken sources.
Analysis: Whole genome sequences and metadata for the 66 isolates were obtained from NCBI. Antimicrobial resistance and virulence gene detection data were obtained from the NCBI Pathogen Detection Isolate Browser using AMRFinderPlus and *fimH* and plasmid sequences were detected using customized workflows using STSR and PlasmidFinder in GalaxyTrakr. Antimicrobial susceptibility testing (AST) data were obtained from the NARMS program, utilizing CLSI standards for broth microdilution using the NARMS standard *E. coli* CMV5AGNF plate and CLSI breakpoints utilized by the NARMS program. AST data were not available from the 15 isolates obtained from published literature. Maximum likelihood trees based on single nucleotide polymorphisms (SNPs) or amino acid changes were created by Dr. Chih-Hao Hsu from FDA/CVM by identifying each of the three virulence genes (*cvaC, iutA, fimH*) in each isolate using the Blast program (Altschul et. al. 1990) and extracting their gene sequences with an in-house script. All gene sequences were aligned using Clustal Omega package (Sievers et. al. 2011) and the maximum likelihood trees were constructed using the PHYLIP package (Felsenstein 1989). Amino acid changes as a result of mutations were evaluated (Shakya et al. 2020).

Table 1. Comprehensive literature review identifying possible virulence factors, mechanisms of action, and genetic targets.

Function	Name	References*
Adhesins	Type I fimbriae	La Ragione, Cooley and Woodward (2000), Vandekerchove D. et al. (2005)
	Type 1 (F1) and P (Pap/Prs) fimbriae for colonization	Dziva F, Stevens MP (2008)
	Stg fimbriae	Lymberopoulos et al. (2006)
	P fimbriae	Kariyawasam and Nolan (2009)
	Autotransporter adhesion AatA	Li et al. (2010); Wang et al. (2011)
	Curli	La Ragione, Cooley and Woodward (2000)
	Temperature sensitive hemagglutinin Tsh	Dozois et al. (2000), Vandekerchove D. et al. (2005), De Carli et al. (2015)
	temperature-sensitive haemagglutinin of imprecise function	Dziva F, Stevens MP (2008), Vandekerchove D. et al. (2005)
	Yqi	Antao et al. (2009)
	E. coli common pilus (ECP)	Stacy et al. (2014)
Antiphagocytic activity/serum resistance	serum resistance	Vandekerchove D. et al. (2005)
	Increased serum survival (Iss)	Nolan et al. (2003), Vandekerchove D. et al. (2005), De Carli et al. (2015)
	Degenerate type III secretion system 2 (ETT2sepsis)	Ideses et al. (2005); Huja et al. (2015)
	O78 LPS	Mellata et al. (2010)
	K and O antigens for anti-phagocytic activity	Dziva F, Stevens MP (2008), Vandekerchove D. et al. (2005)
	K1 capsular polysaccharide	Mellata et al. (2003a)
	TraT	Dziva F, Stevens MP (2008), Vandekerchove D. et al. (2005)
Iron acquisition	iron acquisition systems	Dziva F, Stevens MP (2008), Vandekerchove D. et al. (2005), De Carli et al. (2015)
	Aerobactin operon	Gao et al. (2015a)
	iutA	De Carli et al. (2015)
	Salmochelin	Caza et al. (2008)
	System Sit	Sabri et al. (2008)
	sitA	De Carli et al. (2015)
	Heme utilization/transport protein ChuA	Li et al. (2005); Gao et al. (2012)
Metabolism	Phosphate transport system (*pts*)	Lamarche et al. (2005)
	Nitrite transporter (NirC)	de Paiva et al. (2015)
	Sugar metabolism (Aec35-37)	Chouikha et al. (2006)
Two-component regulatory systems	RstA/RstB	Gao et al. (2015b)
	PhoB/PhoR	Bertrand et al. (2010)
	BarA/UvrY	Herren et al. (2006)
Miscellaneous	SsrA/SmpB	Mu et al. (2013)
	IbeA and IbeB	Germon et al. (2005); Flechard et al. (2012)
	IbeA for invasion	Dziva F, Stevens MP (20080
	Type VI secretion systems	de Pace et al. (2010); Ma et al. (2014)
	Transcriptional regulator (YjjQ)	Li et al. (2008)
	Vacuolating autotransporter toxin (Vat)	Parreira and Gyles (2003)
	Flagella (FliC)	Dziva et al. (2013)
	Group 4 capsule	Dziva et al. (2013)
	iucA	Vandekerchove D. et al. (2005)
	fyuA	Vandekerchove D. et al. (2005), De Carli et al. (2015)
	iroC	Vandekerchove D. et al. (2005)
	colicin or colicin V - common on APEC plasmids	Vandekerchove D. et al. (2005)
	cvaC - gene for colicin and common on APEC plasmids	De Carli et al. (2015)
	colB (colicin B)	De Carli et al. (2015)
	colM (colicin M)	De Oliveira et al. (2020)
	irp-2	De Carli et al. (2015)
	ompT	De Carli et al. (2015)
	hlyF (hemolysin)	De Carli et al. (2015)

Results and Discussion

Of the 66 APEC test strains (Figure 1):

- 100% of retail and cecal isolates were positive for *iutA*, and 83% of clincal isolates. *iutA* was detected in only one positive control, APEC 01, and was not detected in the ExPEC negative control.
- 100% of cecal and clinical isolates were positive for *fimH* and 84% of retail strains. 100% of positive APEC control strains and negative control strains were positive for *fimH*.
- *cvaC* was detected in 25% of clinical isolates and was not detected in positive or negative controls. *cvaC* was detected in 88% of retail and 65% of cecal strains.
- SNPs were not detected in *cvaC*. SNPs were detected in *iutA* and *fimH*, resulting in 1.5-2.5 amino acid changes in *fimH* (Figure 2), with no identified correlation between sources or resistance phenotype.
- 62.7% of the total collection and 75% of clinical colibacillosis strains were pansusceptible.
- 73% of cecal isolates were resistant to ≥1 antimicrobial, with 50% multidrug resistant (MDR)
- Most common MDR pattern – resistance to aminoglycosides, sulfonamides, tetracyclines
- Tetracycline resistance was most common, with 42% of the collection resistant to tetracycline. 61% of cecal and 36% of retail isolates were resistant to tetracycline. The most prevalent antimicrobial resistance gene detected was *tet(A)*, with 36% of retail and 11% of cecal isolates positive for *tet(A)*. Only 1 clinical isolate was tetracycline resistant and was positive for *tet(B)*.
- 57% of cecal and 5% of retail isolates were resistant to sulfonamides, with the *sul1* as the most-commonly detected gene in 8% of retail and 11% of cecal isolates.
- 34% and 16% of cecal and retail isolates and 16% of clinical isolates were beta-lactam resistant. Clinical isolates were only positive for bla_{TEM} (8%) but retail and cecal isolates housed 5 beta-lactamase genes with the most common being bla_{CMY} and bla_{CMY2} (52% of retail and 15% of cecal isolates).
- IncFIB plasmids were detected in 83% of clinical isolates and 94% of retail and cecal isolates. IncFIB plasmids were not detected in positive or negative controls.
- 15 different types of plasmids were identified in this collection of APEC strains, with the second most-commonly detected plasmid being IncFIA.

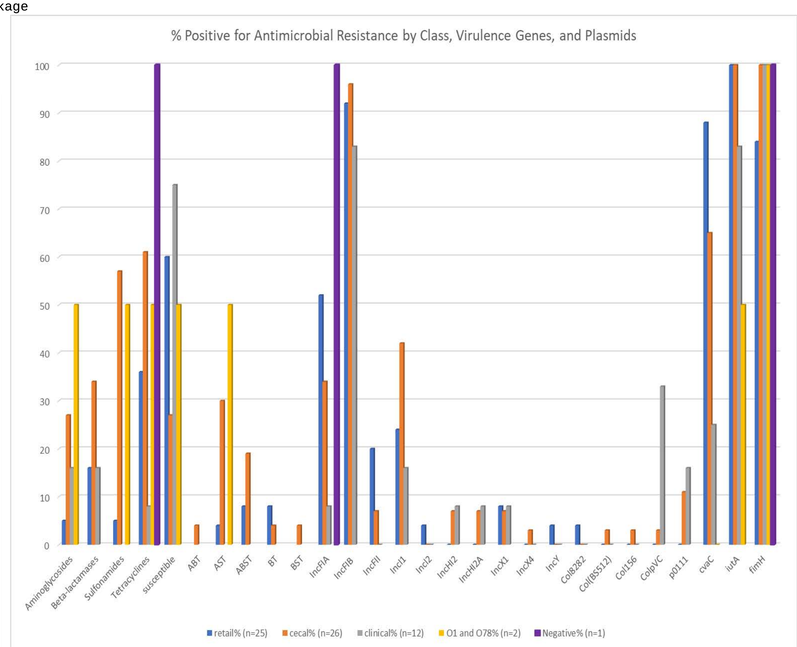

Figure 1. Detection of antimicrobial resistance by antimicrobial class, multidrug phenotype, plasmid, and virulence gene for 66 APEC isolates. ABT (Aminoglycosides, Beta-lactamases, Tetracyclines), AST (Aminoglycosides, Sulfonamides, Tetracyclines), ABST (Aminoglycosides, Beta-lactamases, Sulfonamides, Tetracyclines), BT (Beta-lactamases, Sulfonamides, Tetracyclines)

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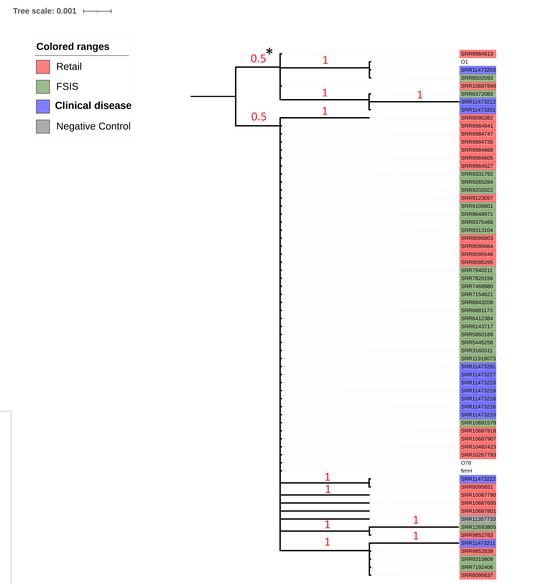

Figure 2. Maximum likelihood tree based on SNPs resulting in amino acid changes in *fimH* for 66 APEC isolates
*0.5 indicates an average of 50% amino acid difference, 1 indicates one amino acid difference

Conclusions

Within our preliminary evaluation, we observed the following trends:

1. Virulence genes are extensively propagated among *E. coli* strains residing in poultry, even if those animals did not break with disease. Failure to break with disease may be a function of the integrity of the animal's immune system, or it could indicate the need for concomitant expression of multiple factors, some of which may not have been included in our preliminary assessment. Future work will include more clinical colibacillosis isolates, as our sample size was small (n=12).
2. The much lower detection of *cvaC* in diseased animals versus healthy animals at slaughter (76% retail and cecal isolates, and 25% in ill animals) was surprising, possibly suggesting alternative genes that are more responsible for colibacillosis disease characteristics. In fact, it would be interesting to see if there are differences in virulence gene expression across the various strains of *E. coli* (e.g., urinary tract, UPEC, and ExPEC).
3. Certain virulence genes appear to be more prone to mutations than others (e.g., SNPs were not detected in *cvaC*, but did cause amino acid changes in *iutA* and more commonly in *fimH*). Future work will investigate correlations between observed virulence gene mutations and co-location on the chromosome or mobile elements with antimicrobial resistance genes by long-read sequencing.
4. Due to the high detection of IncFIB plasmids (94% in retail and cecal strains and 83% clinical strains), this test set may have been unintentionally enriched for plasmids associated with *iss* or *tsh* genetic markers; however, 67% of the collection were pansusceptible, indicating that plasmids associated with the virulence genes studied may not always be associated with resistance genes.
5. It appears that efforts to reduce the use of antimicrobial drugs in poultry have succeeded in constraining the proliferation of resistance genes, since the percent of isolates exhibiting antimicrobial resistance was low in all sources. Additional clinical isolates will be included in future work.
6. Data gaps exist due to the limitations of short read sequencing technologies, the completeness of databases used to screen sequences (PlasmidFinder, AMRFinderPlus), and the limited availability of clinical isolates.