INRS Armand-Frappier Santé Biotechnologie

HIERARCHICAL ROLES OF SERINE PROTEASE AUTOTRANSPORTERS OF *ENTEROBACTERIACEAE* IN THE PATHOGENESIS OF AVIAN PATHOGENIC *ESCHERICHIA COLI* IN TURKEYS.

By

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DEDICATION

To my wife, my life, Pratikshya Paudel Dhakal.

RÉSUMÉ

Les autotransporteurs de sérine protéase des *entérobactéries* (SPATEs) sont des protéines sécrétées qui contribuent à la virulence et fonctionnent comme des protéases, des toxines, des adhésines et/ou des immunomodulateurs. Une souche O1 : K1 *d'E. coli* pathogène aviaire (APEC), QT598, isolée d'une dinde, possède 5 gènes SPATEs : *vat*, *tsh*, *sha, tagB* et *tagC.* Auparavant, Il a été démontré que ces SPATEs jouent un rôle dans un modèle l'infection urinaire chaez le souris. L'objectif global de ce projet est d'analyser les rôles hiérarchiques des gènes SPATE lors d'infections systémiques chez la volaille et l'étalissement d'un modèle d'infection respiratoire chez la dinde). À cette fin, une complémentation en copie unique du gène codant pour les différentes protéines SPATE individuellement ou en combinaison a été générée en insérant les gènes dans le site *att Tn7* du mutant ∆5 SPATEs*.* Les clones générés pour la complémentation et les souches complémentées ont été vérifiés pour l'expression des SPATEs et les phénotypes respectifs. Suite à l'infection des sacs aériens chez la dinde avec la souche QT598 et la dérivée ∆5 SPATEs, une diminution significative de la colonisation des poumons et foies ont été observée. De plus, l'introduction des gènes *sha* et *tsh,* mais surtout c'est deux gènes en combinaison a contribué à un regain de la colonisation des poumons. En effet, la souche ∆5 SPATEs complémentée avec *sha+tsh* ou complémenté avec tous les 5 gènes codant pour les SPATEs ont restauré la capacité d'infection des poumons. De plus, l'analyse de l'expression génique par RT-qPCR dans les poumons et les sacs aériens de dindes infectées par la souche QT598 a montré une augmentation d'expression significatif de *sha et tsh* dans ces tissus respiratoires en comparaison avec la culture in vitro. Dans l'ensemble, les résultats démontrent que les SPATES jouent un rôle cumulatif pour l'infection systémique de la dinde et que Sha et Tsh sont hiérarchiquement plus importants pour la colonisation du système respiratoire.

Mots-clés : *Escherichia coli,* SPATEs, Volaille, Dinde, Infection respiratoire, complémentation, pathogenèse, autotranporteurs, toxines.

ABSTRACT

Serine protease autotransporters of *Enterobacteriaceae* (SPATEs) are secreted proteins that contribute to virulence and function as proteases, toxins, adhesins, and/or immunomodulators. An avian pathogenic *E. coli* (APEC) O1:K1 strain, QT598, isolated from a turkey, has 5 SPATE genes: *vat, tsh, sha, tagB* and *tagC*. These SPATEs were previously shown to contribute to infection in a mouse urinary model. The overall objective of this project was to analyze the hierarchical role of SPATE genes during systemic infections in poultry and establish a turkey air sac infection model. For this purpose, single-copy complementation of genes encoding SPATE proteins individually or in combination were generated by inserting the genes into *att*Tn7 site of ∆5 SPATEs mutant. Clones generated for complementation and complemented strains were verified for the expression of SPATEs and the respective phenotypes. Following air-sac infection in turkeys with wildtype and ∆5 SPATEs mutant of QT598 strain, a significant decrease in bacterial numbers in the lungs and liver were observed. Furthermore, during infection with individual SPATE complemented strains, a regain in infection in lungs was observed with the *sha* and *tsh* complemented strains. Interestingly, infection with *sha-tsh* complemented strain in combination and quintuple (5X) complemented strain significantly restored infective capacity in the lungs. In addition, gene expression analysis using RT-qPCR in the lungs and air-sacs of turkeys infected with wild-type strain QT598 demonstrated upregulated expression of *sha* and *tsh* genes. Altogether, results demonstrate that SPATES collectively contribute to the infective capacity of APEC QT598 in the turkey and that Sha and Tsh are hierarchically important for the infection of by in the respiratory tract.

Keywords: *Escherichia coli,* SPATE, poultry, turkeys, autotransporters, respiratory infection, complementation, pathogenesis, toxins.

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LIST OF ABBREVIATIONS

AIEC	Adherent Invasive *E. coli*
ANOVA	Analysis of variance
DAEC	Diffusely adherent *E. coli*
EAEC	Enteroaggregative *E. coli*
EHEC	Enterohemorrhagic *E. coli*
EIEC	Enteroinvasive *E. coli*
EPEC	Enteropathogenic *E. coli*
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal pathogenic *E. coli*
IBC	Intracellular bacterial community
IBD	Inflammatory bowels disease
InPEC	Intraintestinal pathogenic *E. coli*
LEE	Locus of enterocyte effacement
MS	Mass spectrometry
NMEC	Neonatal meningitis *E. coli*
OMPs	Outer membrane proteins
PAIs	Pathogenicity Islands
SEPEC	Sepsis-associated *E. coli*
SPATEs	Serine protease autotransporters of *Enterobacteriaceae*
ST	Sequence type
T5SS	Type V secretion system
UPEC	Uropathogenic *E. coli*
UTIs	Urinary tract infections

1 INTRODUCTION

Escherichia coli is a Gram-negative bacillus that can infect both humans and animals. Many *E. coli* strains are commensal and reside among the normal intestinal microbiota. However, collectively, some *E. coli* strains have gained the ability to cause a wide range of diseases and illness in a variety of hosts including humans, other mammals, and birds including poultry. In broad terms pathogenic *E. coli* comprises intestinal pathogenic *E. coli* (InPEC) and Extraintestinal pathogenic *E. coli.* (ExPEC) wherein the former causes intestinal infections such as diarrhea and dysentery, and the latter causes extraintestinal infections including urinary tract infections (UTI), meningitis and sepsis in humans, and respiratory tract infections and systemic infections in avian species. Avian pathogenic *E. coli* (APEC) is one of the ExPEC subgroups and is a health concern and economic burden to the poultry industry as it causes a variety of infections in avian species including chickens, turkeys, and ducks (Dho-Moulin *et al.*, 1999; Guabiraba *et al.*, 2015; Mehat *et al.*, 2021). APEC are a diverse group and as with all *E. coli* pathotypes, APEC contain genes that encode virulence factors that contribute to their pathogenic capacity. Our research group has recently discovered and characterized three novel autotransporter proteins TagB, TagC and Sha that belong to the serine proteases known as SPATEs (Serine Protease Autotransporters of Enterobacteriaceae, and in some ExPEC strains. ExPEC (APEC) O1:K1 strain QT598 was isolated from a five-day-old turkey and contains five different SPATE proteins: namely Vat, TagB, TagC, Sha, and Tsh. In this thesis, we are focused on exploring the hierarchical or combined roles of these SPATEs for strain QT598 to cause a systemic infection in an avian experimental infection model. Our hypothesis is that one or a combination of these SPATEs could play more predominant roles during pathogenesis.

In Chapter 2, I have presented my published review article titled "*E. coli* pathotypes their molecular pathogenesis and vaccine strategies". In the same chapter, I have included the literature review of different types of autotransporter proteins (ATs) and elaborated on serine protease autotransporters produced by APEC focusing on their biological context. The article, manuscript chapter presents the research objective of my thesis to interpret the hierarchy of SPATEs in pathogenesis of APEC using a series of specific mutants and complemented strains as tools and an experimental respiratory, air-sac infection model in turkeys.

2 REVIEW ARTICLE AND LITERATURE REVIEW

2.1 Title of review article and authors contribution

The Diversity of *Escherichia coli* Pathotypes and Vaccination Strategies Against This Versatile Bacterial Pathogen

(La diversité des pathotypes d'*Escherichia coli* et des stratégies de vaccination contre cette bactérie pathogène polyvalente)

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2.2 Abstract

Escherichia coli (E. coli) is a Gram-negative bacillus and resident of the normal intestinal microbiota. However, some *E. coli* strains can cause diseases in humans, other mammals and birds ranging from intestinal infections, for example diarrhea and dysentery, to extraintestinal infections, such as urinary tract infections, respiratory tract infections, meningitis, and sepsis. In terms of morbidity and mortality, pathogenic *E. coli* has a great impact on public health, with an economic cost of several billion dollars annually worldwide. Antibiotics are not usually used as first-line treatment for diarrheal illness caused by *E. coli* and in the case of bloody diarrhea, antibiotics are avoided due to the increased risk of hemolytic uremic syndrome. On the other hand, extraintestinal infections are treated with various antibiotics depending on site of infection and susceptibility testing. Several alarming papers concerning the rising antibiotic resistance rates in *E. coli* strains have been published. The silent pandemic of multidrug-resistant bacteria including pathogenic *E. coli* that have become more difficult to treat favor prophylactic approaches such as *E. coli* vaccines. This review provides an overview of the pathogenesis of different pathotypes of *E. coli*, the virulence factors involved and updates on the major aspects of vaccine development against different *E. coli* pathotypes.

2.3 Introduction

Vaccines are a major asset for the reduction of the burden of infectious diseases worldwide. They can provide long term immunity, cheaper modalities than diagnosis and treatment of the infections after they have started, and most importantly - can prevent diseases from occurring in susceptible populations or animal species. Despite the potential benefits of vaccines, we do not see much enthusiasm for the development of vaccines against *E. coli*, particularly for human health. There could be scientific, financial, legal, or political barriers, although increased antimicrobial resistance may promote vaccine development if such pathogens become difficult to treat. Herein, we have summarized aspects of each pathotype of *E. coli* before presenting vaccine strategies, since different antigens or components (inactivated whole cells, O antigen, fimbriae, adhesins, enterotoxins, outer membrane proteins (OMPs)) have been used against different *E. coli* pathotypes. Some of the vaccine strategies have been licensed and shown to be efficacious, but most have only been at the developmental stage, as detailed below.

2.3.1 Escherichia coli

Escherichia coli (*E. coli*) is one of the most intensively studied model organisms in microbiology and molecular biology research (Blount, 2015; Idalia *et al.*, 2017). *E. coli* is a well-known commensal bacterium that is among the first colonizing bacteria of the gut after birth. However, in immunosuppressed patients or in healthy individuals whose physical, anatomical and physiological barriers have been compromised, *E. coli* can cause severe systemic infections (Kaper *et al.*, 2004) (Leimbach *et al.*, 2013). Further, due to the genetic variability some *E. coli* strains are different from the commensal counterparts and encode specific virulence traits that render them capable of causing disease in a variety of animals. Pathogenic *E. coli* are broadly divided into two groups, extraintestinal pathogenic *E. coli* (ExPEC) and intestinal pathogenic *E. coli* (InPEC) (Dale *et al.*, 2015; Denamur *et al.*, 2021; Kaper *et al.*, 2004) (Tenaillon *et al.*, 2010). Depending on the presence of specific virulence factors, mechanisms of infection, tissue tropism, interactions with host cells and clinical symptoms, *E. coli* can be categorised into various pathotypes. These include: (i) Enteropathogenic *E. coli* (EPEC), a cause of acute and prolonged diarrhea in infants; (ii) Enterohemorrhagic *E. coli* (EHEC), which can cause hemorrhagic colitis and hemolytic uremic syndrome (HUS); (iii) Enterotoxigenic *E. coli* (ETEC), a major cause of travelers' diarrhea; (iv) Enteroaggregative *E. coli* (EAEC), a cause of acute and chronic diarrhea; (v) Diffusely adherent *E. coli* (DAEC) which is associated with watery diarrhea in young children; (vi) Enteroinvasive *E. coli* (EIEC), a cause of dysentery and watery diarrhea; (vii) Adherent-Invasive *E. coli* (AIEC) which has been associated in the pathogenesis of inflammatory bowel disease (IBD); (viii) Uropathogenic *E. coli* (UPEC), a common cause of urinary tract infections (UTI); (ix) Neonatal meningitis *E. coli* (NMEC), a top cause of neonatal meningitis; (x) Septicemia-associated *E.* coli (SEPEC), which can cause bacteremia and sepsis; (xi) Avian pathogenic *E. coli* (APEC), which can cause severe respiratory and systemic infections in poultry. *E. coli* can also be classified into distinct phylogenetic lineages: A, B1, B2, D1, D2, E and clade I. Group A mostly represents non-pathogenic *E. coli* that reside along the gastrointestinal tract mucosa. Phylogroup B1 contains both commensal and some strains belonging to the EHEC pathotype. D1, D2, and E represent InPEC. Many ExPEC belong to group B2. *E. coli* strains that are genetically diverse but phenotypically indistinguishable are grouped to cryptic clade I (Baldy-Chudzik *et al.*, 2015; Dale *et al.*, 2015; Köhler *et al.*, 2011; Tivendale *et al.*, 2010). Studies indicate that some APEC and ExPEC strains are phylogenetically closely related and share certain virulence genes (Chaudhuri *et al.*, 2012). APEC and other avian *E. coli* may cause a wide variety of intestinal and extraintestinal infections (Bélanger *et al.*, 2011; Manges, 2016; Matsuda *et al.*, 2010; Meena *et al.*, 2021), and in some cases *E. coli* from poultry

may be a reservoir of human ExPEC and InPEC isolates (Rodriguez-Siek *et al.*, 2005; Spurbeck *et al.*, 2012).

E. coli is one of the most genetically versatile microorganisms and can colonize and persist in primary (bird/animal/human host-associated) and secondary (open or non-host-associated) habitats. The high plasticity of the genome of this bacterial species gives it a tremendous capacity to evolve due to the gain and loss of genes through genetic changes leading to the emergence of pathogenic strains from the commensal strains (Baumgart *et al.*, 2021; Darmon *et al.*, 2014; Dobrindt *et al.*, 2001; Vandecraen *et al.*, 2017). Genomes of pathogenic *E. coli* strains are generally larger, as the pathogenic strains require additional adaptive features including virulence factors. Often, virulence genes are located on transmissible genetic elements such as pathogenicity islands (PAIs), bacteriophages, insertion sequences (ISs), integrons, plasmids, or transposons (Kaper *et al.*, 1999; Sabaté *et al.*, 2006; Torres *et al.*, 2002); hence, they can also be horizontally exchanged and may facilitate novel rearrangements among different bacteria. In contrast, commensal bacteria can also become pathogenic by loss of genes, for example, *Shigella* became virulent by loss of *E. coli* specific genes – such as *cadA* and flagellar genes (Lan *et al.*, 2002; Maurelli *et al.*, 1998). Shiga toxins, enterohemolysin, cytolethal distending toxin, superoxide dismutase, and some outer membrane proteins (OMPs) are some examples of virulence factors encoded by phages in *E. coli* strains (Beutin *et al.*, 1990; Boyd *et al.*, 2002; Schmidt, 2001). The horizontal transfer between different strains favors diversity and versatility resulting in the creation of new pathogenic strains as well as dissemination of acquired virulence genes with novel functions outside their clonal lineage. EHEC acquired *stx* genes (transfer by phages)(Khalil *et al.*, 2016), OI (O-island), and LEE (locus of enterocyte effacement) (PAI) through horizontal gene transfer (Javadi *et al.*, 2017). EPEC emerged by acquisition of the LEE island and *espC* serine protease gene (Kaper *et al.*, 1999). Likewise, there are many identified PAIs in different *E. coli* pathotypes which were required via horizontal gene transfer and can contribute to fitness and the colonization of different niches (Javadi *et al.*, 2017; Messerer *et al.*, 2017).

The accumulating genomic sequence data has led to an increase understanding of *E. coli* virulence factors and mechanisms underlying species diversification and the tracking of foodborne disease outbreaks. In 2011 there was an foodborne outbreak of diarrhea caused by Shiga-toxin-producing *Escherichia coli* (EHEC-STEC-VTEC) O104:H4 in Germany associated with the consumption of raw fenugreek sprouts (King *et al.*, 2012). Interestingly, this strain was more virulent than most Shiga-toxin-producing *E. coli*. DNA sequencing demonstrated that the strain contained toxin-encoding phage similar to 933W phage found in EHEC and also harbored plasmid-borne

virulence factors typically found in EAEC which promote aggregative adherence to intestinal epithelial cells (Beutin *et al.*, 2012; Foley *et al.*, 2013).

Another example is the STEC/UPEC serotype O2:H6 ST141 strain, a STEC with virulence genes *α-hlyA*, *cnf1*, and *clb* common to UPEC (Gati *et al.*, 2019; Gati *et al.*, 2021). This hybrid strain can be a melting pot for pathotype conversion of InPEC and ExPEC because it acquired the *stx*-harboring prophage and ExPEC PAI. It has been also demonstrated that ST141 includes different hybrid versions - UPEC/EAEC, STEC/UPEC/EAEC, and UPEC-Stx/UPEC (Bielaszewska *et al.*, 2014)

2.3.2 Phenomenon of antibiotic resistance

It has been shown that *E. coli* can be highly resistance to many of the antibiotics used by humans since the 1930s (Marshall *et al.*, 2009; Tadesse *et al.*, 2012). This may be due in part to the high rate of gene acquisition and horizontal transfer capacity of *E. coli* strains. The emergence of antibiotic resistance might be multifactorial, but it is largely believed to be caused mainly by human activity and increased antibiotic usage for human health, animal health and food production. (Chokshi *et al.*, 2019; Gopal Rao, 1998; Hasan *et al.*, 2020; Ma *et al.*, 2021; Sengupta *et al.*, 2013). The broadly reported multidrug resistant *E. coli* ST131 is an example of highly virulent ExPEC associated with urinary and bloodstream infections and has promoted the widespread dissemination of *CTX-M-15* gene (Johnson *et al.*, 2010a; Naseer *et al.*, 2009). In general, the *E. coli* strains have evolved to resist major classes of antibiotics such as β-lactams, quinolones, aminoglycosides, sulfonamides and fosfomycin. AmpC-producing *E. coli* strains are dominant in gut colonization of both animals and humans and environmental contamination in developing countries (Ayukekbong *et al.*, 2017; Chokshi *et al.*, 2019; Hassan, 2020; Okeke *et al.*, 1999). As ESBL- and AmpC-producing *E. coli* are increasingly reported as the cause of severe infections (Iseppi *et al.*, 2020; Mikhayel *et al.*, 2021), we are confined to last resource antibiotic classes such as the polymyxins and carbapenems. Also, carbapenem-hydrolyzing oxacillinase-48 (Oxa-48) carrying *E. coli* strains have also been isolated in Europe (Nordmann, 2014) where 134 cases of *E. coli* strains carrying the OXA-48 variant OXA-244 were isolated from clinical samples in Germany (Pfeifer *et al.*, 2012). This same variant was further identified in 119 *E. coli* strains isolated from other European countries (Bakthavatchalam *et al.*, 2016; Dautzenberg *et al.*, 2014; Glupczynski *et al.*, 2012; Liapis *et al.*, 2014; Pitart *et al.*, 2011). Likewise, New Delhi metallo-β-lactamase (NDM-1) and closely related enzymes are a group of zinc-requiring metallo-β-lactamases capable of hydrolyzing a broad range of β-lactams including all penicillins (Williamson *et al.*, 2012), cephalosporins and carbapenems. Further,

resistant strains containing New Delhi metallo-beta-lactamase 1 (NDM-1) as well as over 20 NDM variants have spread and are associated with infections in many parts of the world (Ahmad *et al.*, 2018; Farhat *et al.*, 2020). With the increase in international travel and trade globalization, resistant bacteria have become a worldwide public health threat. Similarly, there is widespread use of antibiotics in food animals for various reasons - growth promotion or ongoing mass prophylactic medication. In many developing countries, there is widespread use of third and fourth generation cephalosporins (ceftiofur and cefquinome) and fluoroquinolones (enrofloxacin) in food animals (Angulo *et al.*, 2009; Collignon *et al.*, 2016; Naveen *et al.*, 2018). The problem is much worse in developing countries due to the increasing number of extended-spectrum β-lactamase-producing and fluoroquinolone-resistant *E. coli* due to lack of regulation, resources, controls, and surveillance (Founou *et al.*, 2017; Hassan, 2020).

Thus, to counteract the above-mentioned antibacterial resistance of highly virulent strains, global efforts are needed to ensure the discovery of alternative solutions. Currently, antimicrobial resistant pathogens are causing 700,000 deaths/year, and 10 million deaths/year are expected by 2050, a number even, higher than the 8.2 million caused by cancer today (Ventola, 2015; Ventola, 2017). Development of novel therapeutic approaches will continue to be essential. In addition, alternative sustainable preventive strategies like vaccination can help in limiting the increase of antibiotic resistant *E. coli*. In the following sections of this review, we will summarize the vaccination strategies to prevent diseases caused by different pathotypes of *E. coli.*

2.4 Diarrheagenic *E. coli* pathotypes

Diarrhea was responsible for the death of at least 370,000 children in 2019; 800,000 fatalities per year according to data of 2013, and second leading cause of death in children under five years old (Dadonaite *et al.*, 2018; Roser *et al.*, 2021). Diarrhea is a global problem, but its high morbidity and mortality occurs among low-income countries. Most of these are caused by InPEC whose members possess distinct virulence traits, different O:H serotypes and characteristic clinical syndromes even though they share some steps in the mechanism of pathogenesis that include attachment to intestinal mucosa and harbor plasmids that encode virulence factors (Rojas-Lopez *et al.*, 2018).

2.4.1 EPEC

Among the pathotypes of InPEC, EPEC is a major cause of infantile diarrhea in developing countries varying from subclinical to fatal infections (Kliegman *et al.*, 2015). The characteristic

histopathological hallmark related to this group of *E. coli* is production of lesions known as “Attaching and effacing (A/E)” lesions that are produced when bacteria intimately attach to intestinal epithelial cells and alter the cytoskeleton through accumulation of polymerized actin beneath the adherent bacteria (Deborah Chen *et al.*, 2005; Donnenberg *et al.*, 1992). EPEC is divided into typical EPEC (tEPEC) and atypical EPEC (aEPEC), based on the presence of EPEC adherence factor plasmid (pEAF) (Hernandes *et al.*, 2009; Trabulsi *et al.*, 2002). They have their own distinct adherence patterns. aEPEC strains exhibit diffuse adherence, localised adherence, and an aggregative adherence pattern, whereas tEPEC strains display a localised adherence pattern. The aEPEC can cause both acute diseases and persistent bloody diarrhea (Kaper *et al.*, 2004). The tEPEC strains are strongly associated with abundant secretory diarrhea with mucus and significant water and electrolyte losses (Deborah Chen *et al.*, 2005; Rojas-Lopez *et al.*, 2018). While aEPEC can be found in both human and animals, the reservoir of tEPEC is human (Trabulsi *et al.*, 2002).

2.4.1.1 Molecular pathogenesis

EPEC pathogenesis involves three steps: i) initial adherence to host cells, ii) translocation of bacterial toxins using a type III secretion system, ii) pedestal formation and intimate attachment (Deborah Chen *et al.*, 2005; Govindarajan *et al.*, 2020; Kaper *et al.*, 2004) (**Figure 2.1**). At first EPEC strains adhere to the enterocytes in the small intestine and form microcolonies in localised or aggregative pattern. This adherence is fostered mostly by a plasmid-encoded, bundle-forming pilus (BFP). It has been shown that BFP mutant strains are less able to cause diarrhea in human volunteers (Bieber *et al.*, 1998; Donnenberg *et al.*, 1997). Initial adherence and microcolony formation are important for EPEC infections, and BFP is one of the key virulence factors for pathogenesis (Girón *et al.*, 1993; Giron *et al.*, 1991; Zahavi *et al.*, 2011).

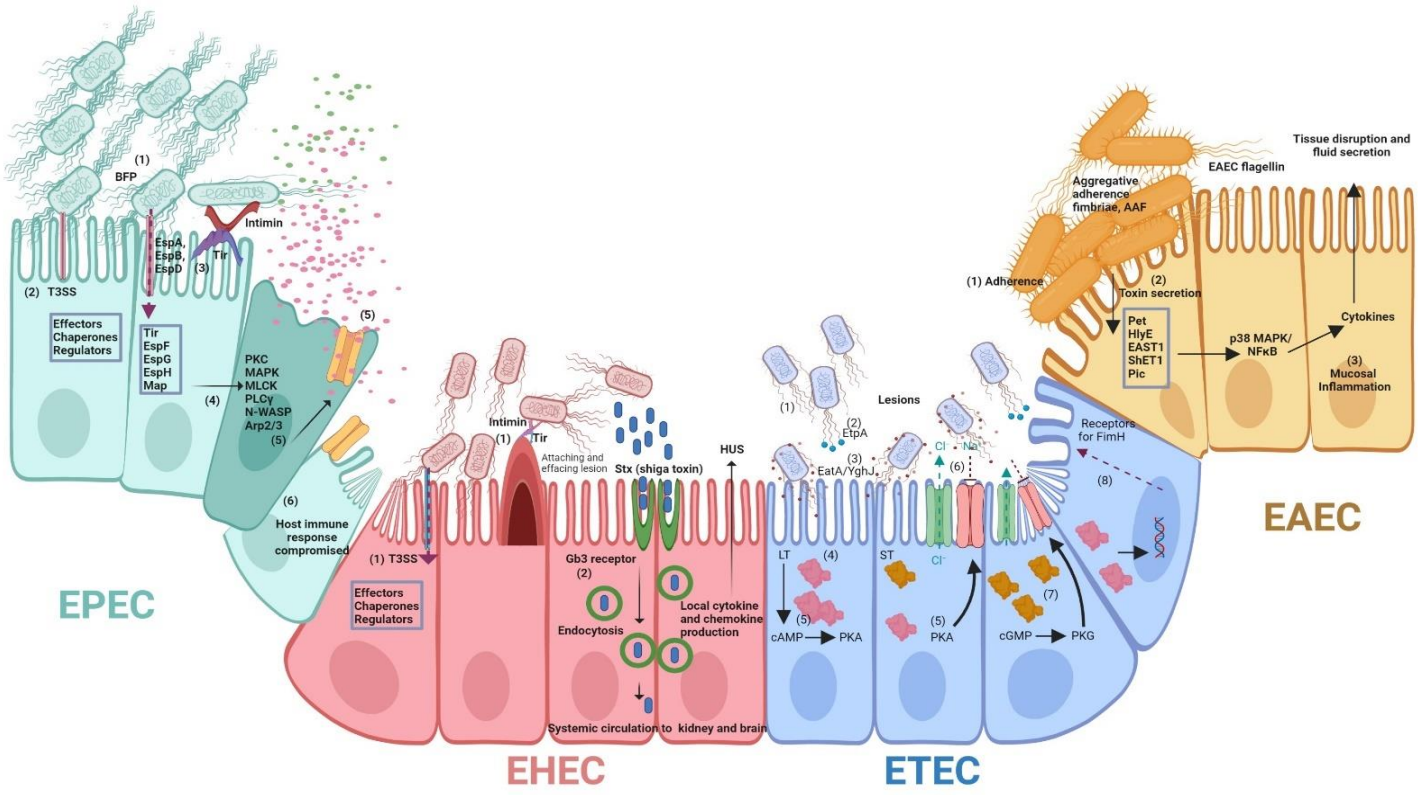

Figure 2.1 Pathogenesis of EPEC, EHEC, ETEC and EAEC.

EPEC (Gray) is famous for production of lesions known as "Attaching and effacing (A/E)" lesions that are produced when bacteria intimately attach to intestinal epithelial cells. Bundle-forming pilus (BFP) helps in interbacterial adherence as well as adherence to epithelial cells (1). Pathogens contact the host cell via T3SS and its effectors (2). The intimate attachment of bacteria with the epithelial cells is mediated by the Tir-EspA-EspB-EspD complex into the host membrane and intimin on the bacterial membrane (3). The type III secretion system releases various effector proteins — including Tir, EspF, EspG, EspH and MAP are migrated in the cytoplasm (4) where it interacts with host proteins such as N-WASP and the Arp2/3 complex to cause actin rearrangement and the pedestal formation. Protein kinase C (PKC), phospholipase Cγ, myosin light-chain kinase and mitogen-activated protein (MAP) kinases are triggered. These multiple complex effacement leads to increase intestinal inflammation, intestinal permeability, and loss of absorptive surface area (5,6). EHEC (Red) is also attaching and effacing (A/E) pathogen that efface the microvilli and subvert host cell actin to form pedestals beneath the attachment site, but the mechanism is slightly different from EPEC, Tir is not phosphorylated. EHEC injects effector proteins such as Tir and EspFu into the host cytoplasm through the T3SS (1) and Tir binds to intimin to intimately attach the bacteria to the host cell (1). Tir and EspFu recruit host factors to subvert host cytoskeleton and actin polymerization. In addition, Shiga toxin (Stx; also known as verocytotoxin) is released in response to stress, further contributing to disease. The B subunit of Stx toxin binds to the glycosphingolipid globotriaosylceramide (Gb3), present in lipid rafts on the surface of the target cell and are internalized (endocytosis) (2), and Shiga toxin is activated through cleavage of the A subunit into two fragments by the protease furin. Stx toxin if absorbed into the systemic circulation, it can cause direct endothelial injury by

increasing inflammation, inducing expression of cytokines and chemokines, and even can damage to important organs, especially the kidney and the brain. ETEC (Blue) strains adhere to intestinal epithelial cells by the help of one or more peritrichous flagella or fibrillar colonization factors (CFs) (1). EtpA mediates the bridging of flagella with glycan receptors present in mucin (2). Mucinolytic serine protease EatA helps to degrade MUC2, the major mucin secreted by goblet cells while YghJ is required for efficient access to the surfaces of enterocytes (3) providing bacterial access to the epithelial surface. Coincident with these events, the bacteria deliver pre-formed LT and ST to their respective receptors on the host cell GM1 gangliosides and GC-C respectively (4), which activates production of cellular cyclic adenosine monophosphate (cAMP) (5), that initiates intracellular signaling cascades that ultimately lead to chloride efflux from CFTR and inhibition of Na+ uptake through the NHE3 Na+/H+ ion exchanger; resulting in the net export of salt and water into the intestinal lumen and diarrhea (6). Heat-stable toxin (ST) binds to guanylate cyclase C to activate the production of cyclic guanosine monophosphate (cGMP), activating protein kinase G (PKG), which phosphorylates ion channel proteins and development of diarrhea (7). LT modulates the transcription of multiple genes including those encoding CEACAMs (Carcinoembryonic Cell Adhesion Molecules), which then serve as receptors for FimH of ETEC expressing type 1 fimbriae that promote ETEC adhesion (8). EAEC (yellow) adheres to small and large bowel epithelia in a thick biofilm, release enterotoxins and cytotoxins, and induce mucosal inflammation. The first step of EAEC infection involves colonization of intestinal mucosa by aggregative adherence fimbriae (AAF) I, II and III which gives characteristic AA pattern as a stacked-brick lattice (1). This is followed by secretion of different enterotoxin and cytotoxins with different functions - Enteroaggregative heat-stable toxin (EAST-1), Plasmid-encoded toxin (Pet), Protein involved in colonization (Pic), Shigella enterotoxin 1 (ShET-1), and Hemolysin E (HlyE) (2). The role of these virulence factors and their clinical outcome is unclear, but they are associated with increased cytokine production and inflammatory markers resulting in mucosal inflammation (3). The figure was created with BioRender.com.

2.4.1.2 Vaccine strategies against EPEC

The aforementioned repertoire of virulence factors which are important for the pathogenesis of EPEC are potential immunogens to stimulate intestinal immune responses and possible targets as vaccine candidates. One report has shown that recombinant *Mycobacterium smegmatis* (Smeg) and *Mycobacterium bovis* BCG strains expressing BfpA or intimin were able to trigger the immune response in mice (Vasconcellos *et al.*, 2012). It is hypothesized that binding of secretory IgA antibody to BFP may interfere with bacterial binding or initial attachment of EPEC and may prevent the downstream signal transduction pathway to manifest diarrhea (Ellis *et al.*, 2003; Loureiro *et al.*, 1998; Mare *et al.*, 2021). Spleen cells grown *in vitro* from recombinant BfpA-immunized mice produced TNF-α and INF-γ. TNF- γ is produced by recombinant intimin (Flores *et al.*, 2002; Vasconcellos *et al.*, 2017). A successful immunization of cattle against an EHEC (*E. coli* O157) strain has been performed with a combination of recombinant EspA, intimin and Tir. In addition, humoral and cellular immune responses are triggered in mice immunized with *Lactobacillus casei* strains constructed to express intimin-β fragments (*L. casei*-Int_{cv}) and immune dominant epitopes of Int280 (McNeilly *et al.*,

2015). These collectively signify virulence factor alone or in combination inside non-infectious immunogenic vectors can work synergistically to protect against EPEC diarrhea (Vasconcellos *et al.*, 2017). List of vaccine projects in different stages against EPEC are listed in **table 2.1**.

Table 2. 1 Reports describing vaccines against Enteropathogenic *E. coli* (EPEC)

Type of vaccine	Component of vaccine	Results/Observations/Outcomes	Animal model (Year)	References
Antigen-Based Vaccine	Recombinant *Mycobacterium smegmatis* (Smeg) and *Mycobacterium bovis* BCG to express BfpA or intimin.	Yielded high titer of IgG and IgA antibodies in serum of immunized mice. Mice immunized with recombinant BfpA showed TNF-α and INF-β, and TNF-α only with recombinant intimin.	Mice immunized by oral gavage or intraperitoneal injection (2012)	(Vasconcellos *et al.*, 2012)
	Combination of purified recombinant EspA, Intimin, and Tir.	Showed protection of immunized cattle against O157 challenge.	Male Holstein-Friesian calves immunized orally (2010)	(McNeilly *et al.*, 2010)
	Lactobacillus casei expressing intimin-β and immune-dominant isotopes of Int280.	Induced cellular and humoral responses in mice. Serum antibodies inhibited EPEC adhesion to epithellial cells in vitro.	Mice immunized intranasally (2008)	(Ferreira *et al.*, 2008)
Plant-based vaccine	Transgenic plants expressing intimin, and BfpA	Proposed edible vaccines under strategic and regulation planning	Mice immunized orally (2002)	(da Silva *et al.*, 2002)
Live-attenuated bacterial vaccine	Live attenuated *ΔespFΔushA Citrobacter rodentium* strain	Oral administration in mice yielded efficient systemic and humoral immunity against *C. rodentium* virulence factors	Mice immunized by oral gavage (2022)	(Wang *et al.*, 2022b)

Adjuvanted whole-cell vaccine	Cholera toxoid (CTB)-adjuvanted formalin-killed whole bacterial cell (EPEC)	100% survival rate of Balb/C mice when challenged with EPEC	Mice immunized intraperitoneally (2016)	(Gohar *et al.*, 2016)

2.4.2 EHEC

EHEC strains are Shiga-toxin encoding *E. coli* (STEC), also called as verotoxin producing *E. coli* (VTEC) that infect the alimentary tract and cause hemorrhagic diarrhea (Welinder-Olsson *et al.*, 2005), hemorrhagic colitis and hemolytic-uremic syndrome (HUS) that can result in kidney failure and neurological complications in humans (Karpman *et al.*, 2015; Scheiring *et al.*, 2008). Unlike EPEC which predominantly infects the small intestine, EHEC colonizes the large intestine (Frankel *et al.*, 1998). EHEC are mainly responsible for severe cases of foodborne infection out of >200 known serotypes of EHEC, O157:H7 have been associated with most foodborne outbreaks (Bavaro, 2012; EA, 2002). It was first isolated from undercooked minced meat after multi-state outbreak in 1982 in the United States (Meng *et al.*, 2012). Because of multiple outbreaks, easy transmission, and complications for the use of antibiotics for EHEC treatment (Smith *et al.*, 2012; Tarr *et al.*, 2005); this pathotype represents a major public health issue (Braeye *et al.*, 2014; Gaspar *et al.*, 2014; Kanayama *et al.*, 2015; Manitz *et al.*, 2014; Meagher, 2022). EHEC naturally resides in the intestine of ruminant animals, and zoonotic transmission occurs after the consumption of contaminated animal products (mainly ground beef), contaminated water (cross-contaminated from beef), improperly cooked meats and inefficiently washed fruits and vegetables (Karpman *et al.*, 2015; Meng *et al.*, 2012; Welinder-Olsson *et al.*, 2005). In the United States, an estimated 63000 cases of hemorrhagic colitis caused by EHEC are reported annually (Scharff, 2012). HUS is a leading cause of acute renal failure in children past two decades (Trachtman, 2013; Williams *et al.*, 2002). While there is an increased burden of EHEC outbreaks mainly affecting developed countries, a EHEC-EAEC hybrid strain also caused a serious outbreak in Europe with 3816 reported cases that included 54 deaths and 845 HUS cases (Prager *et al.*, 2014; Santos *et al.*, 2020).

2.4.2.1 Molecular pathogenesis

Stx toxins, also known as verotoxins (VT), are the key virulence factor of EHEC. The Shiga toxin family is composed of Stx1 which is nearly identical to the Shiga toxin of *Shigella dysenteriae* and differs only at a single amino acid whereas Stx2 shares less than 60% amino acid homology to Stx1 (Karpman *et al.*, 2015; Nguyen *et al.*, 2012; Welinder-Olsson *et al.*, 2005). Stx consists of five identical B subunits and are soluble toxins (Meng *et al.*, 2012). After bacteria release these powerful cytotoxins, they are translocated from gut lumen to nearby tissues and bloodstream. The holotoxin binds to the glycolipid globotriaosylceramide (Gb3) on the target cell surface and toxin gets internalized where a single A subunit cleaves ribosomal RNA and halts host cell protein synthesis. Stx can also induce apoptosis in intestinal epithelial cells, cause local damage in the colon resulting in hemorrhagic colitis, necrosis and perforation of the intestine (Karch, 2001; Tesh *et al.*, 1991). EspP is a serine protease autotransporter, which contributes to biofilm formation by forming macroscopic rope-like polymers that are refractory to antibiotics and mediates adherence to host cells and cytopathic effects (Xicohtencatl-Cortes *et al.*, 2010). EspP can also cleave host coagulation factor V and serpins which can result in prolonged hemorrhage and contribute to EHEC pathology (Dutta *et al.*, 2002; Tse *et al.*, 2018; Weiss *et al.*, 2012).

2.4.2.2 Vaccine strategies against EHEC

In the past two decades, EHEC has been the most studied *E. coli* pathotype for vaccine development mainly due to the severity of infection with increased number of HUS cases, and issues preventing use of certain antibiotics to treat STEC (Smith *et al.*, 2012). Unlike other pathotypes, use of antibiotics exacerbates STEC disease as antibiotics can induce EHEC to increase production of Shiga toxins and release more toxins during bacterial cell lysis. Since EHEC can colonize the intestine of animals, there have been successful experiments to assay the colonization ability of EHEC in different animal models ranging from neonatal calves (An *et al.*, 2000; Dean-Nystrom *et al.*, 1997), germ-free piglets (where they also show CNS symptoms like the severe infection in humans) (Dean-Nystrom *et al.*, 2000; Tzipori *et al.*, 1995), ferrets (oral infection model) that develop HUS following O157 infection, to different murine models (intra-gastric inoculation model) (Conlan *et al.*, 1998; Karpman *et al.*, 1997; Taguchi *et al.*, 2002; Woods *et al.*, 2002). The availability of a wide range of animals provides valuable models to investigate host immune response following EHEC infection as well as for vaccine development.

To date, different strategies like Shiga toxin-based vaccination to neutralize the effect of toxin by using antibodies engineered against the A subunit of Stx2 and B subunit of Stx1, Stx toxoids

(Bitzan *et al.*, 2009; Mejías *et al.*, 2016), attenuated bacteria- where the LEE-encoded regulator (Ler) which regulates genes inside, and outside pathogenicity island region is deleted or disrupted making the bacteria non-pathogenic but immunogenic enough to impede the pathologic effects of the toxin (Liu *et al.*, 2009). In addition, other methods like bacterial ghosts where bacteria are engineered to have controlled expression of a lysis gene that lyses the bacteria and form empty bacterial cell envelopes with the composition of the cell envelope of living cells which are non-infectious but induce the mucosal immune response. Other protein-based, peptide-based, plant-based, DNA-based, polysaccharide-based, or adjuvant enhanced vaccines have been reported to successfully induce the host immune response and decrease lethality of EHEC in different animal models, and are summarized in **table 2.2** (Rojas-Lopez *et al.*, 2018).

The attaching and effacing (A/E) family of gastrointestinal bacterial pathogens includes EPEC and EHEC. Both EPEC and EHEC have high homology in their LEE and O islands (Schmidt, 2010). Virulence of these pathotypes depends on a T3SS and equivalent secreted proteins like EspS and intimin (Rojas-Lopez *et al.*, 2018). From epidemiological data, it has been observed that EHEC occurrence is significantly lower in the region where EPEC is endemic (Calderon Toledo *et al.*, 2011; Garrine *et al.*, 2020). This strategy has been exploited by researchers to utilize the attenuated EPEC O126:H6 where EspB and intimin antibodies from EPEC vaccination were cross-reactive with EspB and intimin from EHEC and showed reduced mortality in mice following an EHEC infection (Calderon Toledo *et al.*, 2011). These vaccines are summarized in **table 2.2**.

Table 2.2 Reports describing vaccines against Enterohemorrhagic *E. coli* (EHEC)

Type of vaccine	Component of vaccine	Results/Observations/Outcomes	Animal model (Year)	References
Attenuated bacterial vaccines	Attenuated *Salmonella enterica* Typhimurium expressing recombinant EspA, intimin and Stx2B	Significantly higher antibody titers against EspA, intimin and Stx2B, and specific lymphocyte proliferation.	Mice immunized orally (2011)	(Gu *et al.*, 2011)
	γ-intimin variant expressed by attenuated *Salmonella enterica* Typhimurium χ3987	In mice: Increased IgG in serum and IgA in feces. Reduced EHEC O157:H7 shedding and colonization post-challenge.	Oral immunization of mice (2012)	(Rojas-Lopez *et al.*, 2018)

	(*Δcya,Δcrp,Δasd*) and H683 (*ΔaroΔasd*)			
	Attenuated EPEC O126:H6	Reduced mortality in EHEC challenged mouse model and cross-reaction against EspB and intimin EPEC antibodies with EspB and intimin from EHEC.	Mice immunized orally (2016)	(Cepeda Molero, 2016)
	Recombinant bacillus Calmette-Guérin expressing Stx2B (rBCG-Stx2B)	Significant levels of Stx2 IgG in mice. Higher survival rate (>65%) of immunized mice challenged with EHEC.	Mice immunized orally (2012)	(Fujii *et al.*, 2012)
	EHEC O157:H7 86-24 strain *ΔlerΔstx2* expressing Stx1A Stx2A detoxified	Lower colonization of EHEC O157:H7 after challenge.	Oral immunization of mice (2009)	(Liu *et al.*, 2009)
Shiga toxin-based vaccines	cαStx1B and cαStx2A antibodies.	Safety and good tolerance in a human trial single-dose study	Human volunteers (2009)	(Bitzan *et al.*, 2009)
	one anti-serum albumin VHH and two copies of anti-Stx2B VHH.	Decreased toxicity of EHEC in Stx2 lethal mouse model	Mice immunized orally (2016)	(Mejías *et al.*, 2016)
Bacterial ghost-based vaccines	Bacterial ghosts of O157:H7 which is unable to cause infection	Anti-toxicity effect on Vero cell culture. Reduced colonization of EHEC O157:H7 and 93% and 100% survival in orally and rectally immunized mice respectively.	Orally and rectally immunized mice (2015)	(Cai *et al.*, 2015)
	Stx chimeric protein exposing bacterial	Increased IgG and IgA antibody titers to Stx1A and Stx2B.	Intranasal immunization of mice (2012)	(Mayr *et al.*, 2012)

	ghosts of O157:H7 (Stx2Am-Stx1B)	Survival rate >50% in immunized mice.		
Peptide-based vaccines	Peptide KT-12 (KASITEIKADKT) conjugated with KLH	Elevated levels of IgG in subcutaneously immunized mice and IgA in intranasally immunized mice.	Intranasal immunization f mice (2011)	(Zhang *et al.*, 2011)
	C terminal region of intimin	Reduced bacterial adherence to Hep-2 cells and confers protection in immunized mice	Oral immunization of mice (2011)	(Wan *et al.*, 2011)
Protein-based vaccines	EspA-Stx1A fusion protein-based vaccine	Crude toxin Stx2 challenged mice showed 95% survival with high titers of IgG to EspA-Stx1A in treated mice.	Oral immunization of mice (2009)	(Cheng *et al.*, 2009)
	Stx1B-Stx2-truncated intimin fusion protein	EHEC O157:H7 challenged immunized mice had 100% survival rate.	Mice model (2009)	(Rojas-Lopez *et al.*, 2018)
Plant-based vaccines	Cell line from *Nicotiana tabacum* (tobacco) NT-1 that expresses inactivated Stx1A.	Stx2-specific IgA in feces of orally immunized mice, and protection against STEC with more than 75% survival rate.	Orally immunized mice model (2018)	(Rojas-Lopez *et al.*, 2018)
	Five recombinant EHEC proteins including NleA, Stx2b, and EspA expressed from *Nicotiana benthamiana* and transplastomically in *Nicotiana tabacum*.	Immunized sheep with leaf tissue (feeder) showed less shedding of EHEC O157:H7 when challenged.	Sheep (2018)	(Rosales-Mendoza *et al.*, 2018)

Adjuvant improved vaccines	Adjuvanted EspB and/or C-terminal of γ-intimin protein with MALP-2.	Significantly higher titers of IgA in immunized mice.	Orally immunized mice (2013)	(Garcia-Angulo *et al.*, 2013)
	Chimeric Tir-Stx1B-Stx2B adjuvanted with Zot.	Significant increased IgA and IgG and reduced bacterial shedding in feces post-challenge in subcutaneously immunized mice. Partial protection against EHEC.	Subcutaneously immunized mice (2019)	(Khanifar *et al.*, 2019)
Polysaccharide-based vaccines	O-specific polysaccharide of EHEC O157:H7 conjugated with recombinant exotoxin A of *P. aeruginosa*	Elevated IgG against LPS in vaccinated children with non-collateral reactions to the vaccine.	Human volunteers (2014)	(Szu *et al.*, 2014)
DNA-based vaccines	Stx2AΔAB DNA vaccine	Immunized mice showed partial protection when challenged with native Stx2. Toxin neutralization is observed in Vero cell culture.	Intranasally immunized mice (2009)	(Rojas-Lopez *et al.*, 2018)
	C-terminal domain of *EscC*	Increased IgG in sera and IgA in feces of immunized mice. Reduced bacteria in feces, colon, and cecum post-challenge with EHEC.	Orally immunized mice (2014-2016)	(García-Angulo *et al.*, 2014; Tapia *et al.*, 2016)
	pVAX-efa1 (*efa-1'*)	Significantly elevated levels of specific mucosal IgA and reduced EHEC colonization post-challenge.	Intranasally immunized mice (2016)	(Riquelme-Neira *et al.*, 2016)

2.4.3 ETEC

The ETEC pathotype is one of the principal causes of acute “travelers’ diarrhea”, affecting tourists visiting low-income countries, and is predominant in areas with poor sanitation and inadequate clean water (Okoh *et al.*, 2008; Qadri *et al.*, 2005). ETEC infections are often characterised by diarrheal illness ranging from mild and self-limiting to cholera-like symptoms (Vicente *et al.*, 2005). They are defined by production of heat-labile (LT) and heat-stable (ST) enterotoxins, which disrupt ion secretion mechanisms in the intestine leading to watery diarrhea (Fleckenstein *et al.*, 2010; Fleckenstein *et al.*, 2019). Globally there are millions of cases of ETEC infection and over tens of thousands of deaths annually in developing countries among children less than 5 years old (Hosangadi *et al.*, 2019; Khalil *et al.*, 2021). ETEC infections are also a major cause of traveller`s diarrhea with more than one million annual cases worldwide. In the United States alone there are 40,000 estimated cases of ETEC infection annually (McGregor *et al.*, 2015; Olson *et al.*, 2019; Steffen, 2005).

2.4.3.1 Molecular pathogenesis

ETEC strains adhere to the small intestinal mucosa with the help of one or more proteinaceous pili/fimbriae also called colonization factors (CFs) (Girón *et al.*, 1994). Following initial adhesion and colonization, ETEC cause diarrhea not by invading the mucosa but by producing plasmid-encoded heat-labile (LT) and/ or heat-stable (ST) enterotoxin (**Figure 2.1**). LTs are closely related in structure and function to cholera enterotoxin produced by *Vibrio cholerae* (Spangler, 1992). LTs increase host intracellular cAMP through activation of a cAMP-dependent kinase and activate the main chloride channel of epithelial cells resulting in increased chloride secretion from crypt cells. This ion imbalance causes alteration of electrolyte homeostasis, resulting in loss of fluids from tissue and subsequent diarrhea (Nataro *et al.*, 1998). ETEC is also an important veterinary pathogen associated with post-weaning diarrhea in both pigs and cattle, and STb toxin is involved in diarrhea (Khan *et al.*, 2006; Nataro *et al.*, 1998). ETEC also secrete plasmid encoded virulence factors EatA and EtpBAC. EatA is a serine protease autotransporter of Enterobacteriaceae (SPATE) which can cleave substrates identified for cathepsin G (Patel *et al.*, 2004) and EtpBAC is an extracellular adhesin which is a 2-partner secretion system responsible for the export of EatA. The *yghJ* gene present in the chromosome upstream of genes encoding the type II secretion system of multiple ETEC isolates encodes another antigen YghJ (SslE). EatA and YghJ both degrade MUC2 mucin secreted by goblet cells of small intestinal epithelia and facilitate bacteria to overcome the mucin barrier and release of ETEC enterotoxins upon host enterocytes (**Figure 2.1**).

2.4.3.2 Vaccine strategies against ETEC

Because of the substantial morbidity and mortality in the paediatric population and long-term consequences of enteric infections on child growth and development, vaccination could be a promising means of ETEC prevention. In the past two decades different strategies were developed and employed for ETEC vaccine development either to prevent attachment of ETEC to the proximal small intestinal mucosa or to protect via immunity, through IgA antibodies directed against LT (Khalil *et al.*, 2021; Rojas-Lopez *et al.*, 2018). Among these strategies ETVAX is the most advanced candidate which is currently in Phase 2b clinical trials (Qadri *et al.*, 2020). This strategy utilizes inactivated whole cell (one *E. coli* K-12 and three O78 positive *E. coli*) vaccine strains that overexpress CFA/I, CS3, CS5 and CS6 antigens combined with hybrid LT/CTB (B subunit BS of cholera toxin) with and without dmLT adjuvant (Clements *et al.*, 2018; Norton *et al.*, 2011). ETEC strains are heterogenous exhibiting different O:H serotypes and to provide broad spectrum protection, various fimbrial antigens present in the most prevalent ETEC pathotypes have been used in this strategy. This vaccine demonstrated excellent safety in an age-descending trial in Bangladesh, and the inclusion of the dmLT adjuvant increased the magnitude and kinetics of mucosal antibody responses in both Bangladeshi infants and Swedish adults (Qadri *et al.*, 2020). Theoretically, a multivalent ETEC vaccine which can express CFA/I, CFA/II and CFA/IV antigens can protect against the majority of ETEC strains worldwide (Barry *et al.*, 2019b; Leach *et al.*, 2017; Lundgren *et al.*, 2014).

Another vaccine that is further along in the development is live attenuated *E. coli* expressing ETEC fimbriae. *E. coli* strain E1392-75-2A is a prototype demonstrated in clinical trials that expresses CS1 and CS3 fimbriae but lacks genes that encode LT and ST (Levine *et al.*, 2020; Turner *et al.*, 2011; Turner *et al.*, 2001). This strain was derived in the Central Public Health Laboratory, London, U.K., wherein the genes encoding LT and ST were spontaneously deleted from the CFA/II plasmid. All volunteers who received 10^{10} CFU doses of strain E1392-75-2A developed significant rises in intestinal fluid SIgA antibody to CS1 and CS3 and the vaccinees were significantly protective ($p<0.005$, 75% vaccine efficacy) against ETEC challenge strain E24377A (O139:H28)(Barry *et al.*, 2016). However, around 15% of the subjects had mild diarrhea after taking this live oral vaccine. On the same lines, live attenuated vectors like *Shigella* and *Salmonella* have been used to express ETEC fimbrial antigens and LT antigens (Barry *et al.*, 2019a; Tse *et al.*, 2018). An attenuated *Shigella* strain expressing ETEC CFA/I and CS3 fimbriae elicited SIgA mucosal antibody responses to those antigens in a guinea pig model (Koprowski *et al.*, 2000; Svennerholm *et al.*, 2016). A multivalent live oral vaccine against both *Shigella* and ETEC is being developed and is in Phase 1 clinical trial known

as a ShigETEC toxin hybrid which is LPS-free cell expressing conserved ETEC and *Shigella* antigens (Girardi *et al.*, 2022). Likewise, a *Shigella* hybrid (1208S-122) is also in Phase 1 trial which is an attenuated *Shigella* vaccine strain engineered to express ETEC CF and LT (Khalil *et al.*, 2021). If this attenuated *Shigella* expresses ETEC fimbrial colonization factors and genetically detoxified LT, protection is undeniable. Further, a fimbrial tip adhesin-based vaccine, a multiple epitope fusion antigen (MEFA) based vaccine, and protein-based vaccines against ETEC virulence factors are also in various preclinical phases of testing (Rojas-Lopez *et al.*, 2018). These different ETEC vaccine candidates are listed in the **table 2.3**.

Table 2.3 Reports describing vaccines against Enterotoxigenic *E. coli* (ETEC).

Type of vaccine	Component of vaccine	Results/Observations/Outcomes	Animal model/ Phase (Year)	References
Attenuated bacteria-based vaccines	Attenuated ETEC E1392/75-2A *ΔaroCΔompR* and ETEC E1392/75-*2AΔaroCΔompR ΔompC* mutations	Significant yield in IgA and IgG and CS1 and CS3 specific antibodies.	Mice immunized intranasally (2001)	(Turner *et al.*, 2001)
	ETVAX (attenuated bacteria expressing CS6 in *E. coli* K-12 and CFA/I, CS3, CS5 in ETEC O78 toxin-negative) with LCTBA hybrid protein and mutated Heat-lable (LT)	Currently in clinical trials (NCT02531802). High titers of fecal, jejunal and serum IgA and IgG in orally immunized humans	Phase II clinical trials (2020)	(Akhtar *et al.*, 2019; Seo *et al.*, 2020)
	ACE527 ETEC complex (ACAM2022 (O141:H5, expressing CS5 and CS6), ACAM2025	33-98% protection in reducing the duration of diarrhea in human clinical trials (NCT00901654)	Phase I clinical trials (2015)	(Harro *et al.*, 2011; Turner *et al.*, 2011)

	(O39:H12, expressing CFA/I) and ACAM2027 (O71:H-, expressing CS2, CS3, and CS1)			
	Attenuated ETEC strains expressing CFA/I, CS2 and CS3 and CS1, CS2, and CS3 generating ACAM2010, 2007, 2017 strains respectively	Elevated levels of IgA against CFA in orally immunized human volunteers	Human volunteers, double blind trials (2008)	(Turner *et al.*, 2011)
	Attenuated ETEC strains expressing CS5, CS6, LT, ST and EAST1 generating ACAM2025, 2022, 2027 strains.	Double-blind placebo-controlled Phase II challenge trial	Phase II clinical trials (2019) NCT01739231	(Chakraborty *et al.*, 2019; Tobias *et al.*, 2008)
	Strains of *Vibrio cholerae* expressing CFA/I	Significantly yielded high levels of IgA and IgG titers in serum of immunized mice.	Mice immunized orally (2008)	(Tobias *et al.*, 2010)
	Non-toxigenic *E. coli* expressing CS2, CS4, CS5, or CS6 and CFA/I	Significantly induced IgG+IgM and IgA antibodies CS6 in sera and in feces respectively in immunized mice	Oral immunizatin of mice (2010)	(Roy *et al.*, 2010; Roy *et al.*, 2009; Roy *et al.*, 2011)
Adhesin-based vaccines	Recombinant ETEC expressing two-partner secretion protein A (EtpA)	Less bacterial (ETEC) colonization in gut of immunized mice	Intranasal immunization of mice model (2009-2016)	(Zhang *et al.*, 2017)

	CS21/LngA formulated with cholera toxin	Increased specific IgG and IgA in serum and feces and intestinal lavages respectively. Reduced shedding in immunized mice	Intranasal immunization of mice (2017)	(Leitner *et al.*, 2015)
OMV-based vaccines	ETEC OMVs *ΔmsbBΔeltA*	Detoxified OMVs induced higher titers of IgG1, IgM, and IgA and significantly reduced wild-type ETEC colonization in immunized mice	Mice immunized intranasally (2015)	(Leitner *et al.*, 2015)
	Vibrio cholerae OMVs *ΔmsbBΔctxABΔflaA* expressing ETEC FliC and CFA/I	OMVs yielded higher titers of IgG1, IgM, and IgA and reduced wild-type ETEC colonization and spread in immunized mice	Mice immunized intranasally (2015)	(Harris *et al.*, 2011)
Autotransporter-based vaccines	Recombinant Ag43 and pAT (autotransporters)	Significant increase in fecal IgA and partial protection against intestinal colonization of ETEC in immunized mice.	Mice immunized intranasally (2011)	(Behrens *et al.*, 2014)
Toxin-based vaccines	Heat-labile (LT) toxin using skin patch	Yielded significant levels of anti-LT IgG and IgA in 97–100% of human volunteers. Currently in Phase 2 clinical trial completed phase (NCT00565461).	Phase II clinical trial (2020)	(Rahjerdi *et al.*, 2019)
	STaP13F-LTR192G toxoid fusion protein	Induced IgG specific antibodies for LT and STa in serum and feces and IgA in feces in immunized mice.	Oral immunization of mice (2019)	(Turner *et al.*, 2001)

2.4.4 EIEC

EIEC strains show similar genetic, biochemical and pathogenic characteristics to *Shigella,* for example, the ability to invade the mucosa of the colon (Pasqua *et al.*, 2017; Ud-Din *et al.*, 2014). Unlike other pathotypes such as EPEC, EHEC, and ETEC that replicate and infect extracellularly; EIEC strains penetrate the mucosa, after invading epithelial cells, and can replicate and migrate within epithelial cells and macrophages as well. A large (>200kb) virulence plasmid (pINV) found in EIEC and all *Shigella* species encodes genes required for pathogenesis (Lan *et al.*, 2004; Lan *et al.*, 2002; Pasqua *et al.*, 2017). This pathogenesis is known to be the result of multiple effects of the plasmid-borne type III secretion system encoded by the *mxi-spa* locus that secretes invasin proteins including IpaA, IpaB, IpaC and IpgD for signalling events and other actions (Croxen *et al.*, 2010; Kaper *et al.*, 2004). Although EIEC infections are reported in countries with poor sanitation, hygiene and socioeconomic status, there have been EIEC outbreaks in the USA, Japan, Israel and recently in Italy in 2012 and Nottingham, UK in 2014 (Dhakal *et al.*, 2019; Herzig *et al.*, 2019; Lagerqvist *et al.*, 2020; Newitt *et al.*, 2016). Most of these outbreaks are sporadic and travel related. These outbreaks signify the ability of EIEC to induce gastrointestinal disease outbreak across the world.

2.4.4.1 Molecular pathogenesis

During infection EIEC strains initially penetrate and enter into epithelial cells (Govindarajan *et al.*, 2020), lyse the endocytic vacuole, multiply inside the cells and migrate to adjacent cells through the cytoplasm (**Figure 2.2**). During initial penetration T3SS insert a pore containing IpaB and IpaC into host cell membranes (Govindarajan *et al.*, 2020). IpaC induces actin polymerization and activates GTPases Cdc42 and Rac which leads to the formation of cell extensions. Actin polymerization and lamellipodial extensions of host cells are induced by IpaC. In the same line, IpaA alter cell extensions induced by IpaC by binding to vinculin and inducing actin depolymerisation leading to a structure that mediates bacterial cell entry. IpgD helps the internalization by inducing host cell membrane blebbing (Dhakal *et al.*, 2019; Govindarajan *et al.*, 2020; Prats *et al.*, 1995; Schnupf *et al.*, 2019). Multiple factors including T3SS effectors such as IpaB and IpaC, which are used for phagosomal escape by bacteria; host factors such as Rab5 and Rab11; and other cytosolic access factors utilised by intracellular bacterial pathogens are involved in rupturing the endocytic vacuole in less than 10 minutes following cell entry (Kaper *et al.*, 2004; Rojas-Lopez *et al.*, 2018; Schnupf *et al.*, 2019). After this, bacteria multiply inside cells and invade and damage surrounding epithelial cells that results in Shigellosis like symptoms such as watery diarrhea with mucus, with

leukocytes and blood in the stool, abdominal pain with cramps and tenesmus, fever, and systemic toxicity as well (Kotloff *et al.*, 2018; Lambrecht *et al.*, 2022; Schnupf *et al.*, 2019).

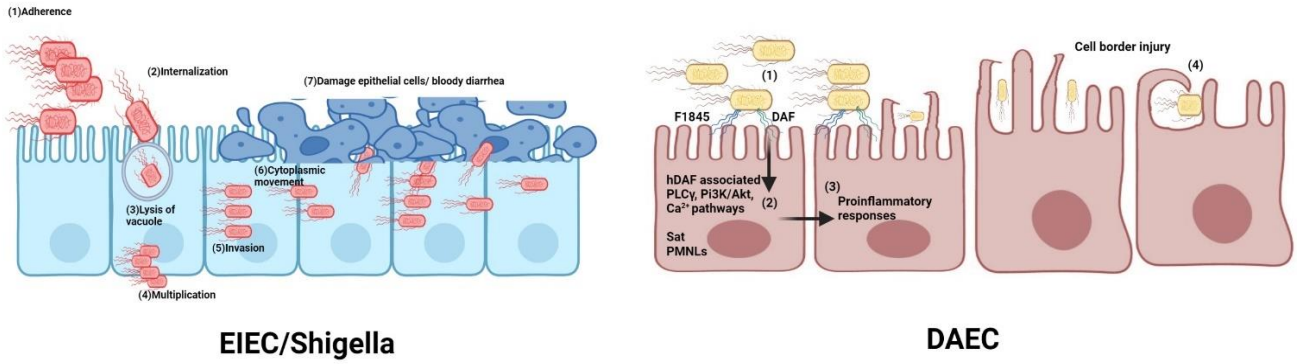

Figure 2.2 Pathogenesis of EIEC/*Shigella* and DAEC

show similar types of pathogenesis. EIEC adhere to the basolateral surface of enterocytes and form a pore in the host membrane (1). Invasion plasmid antigens (IPAs) mediate the uptake of EIEC into enterocytes (2), which is followed by a process of host cell entry (3), multiplication (4) and intercellular spread of EIEC (5,6), ultimately leading to death of the enterocyte (7). Following escape from the entry vacuole (3), Shigella/EIEC drive actin polymerization at one pole through IcsA-dependent recruitment of N-WASP and ARP2/3. This allows for intracellular motility. Invasive bacteria released from damaged enterocytes induce another wave of uptake by phagocytosis in healthy cells and then repeat the cycle of survive and replicate or disseminate from cell to cell via an actin-based motility process. DAEC are characterized by the diffuse adherence pattern on cultured epithelial cells. Around 75% of DAEC harbor adhesins from the Afa/Dr family, responsible for this adherence phenotype. Infection starts with interaction of Afa/Dr family of adhesins with membrane bound receptors, including decay-accelerating factor (DAF) by Afa/Dr/DAF adhesins (AfaE-I, AfaE-II, AfaE-III, AfaE-V, Dr, Dr-II, Nfa-1, and F1845) (1). A signaling pathway involving protein tyrosine kinase(s), phospholipase Cγ, phosphatidylinositol 3-kinase, protein kinase C, and an increase in [Ca2+] that controls the rearrangements of brush border-associated F-actin induce structural changes of microvilli (2). Sat, serine protease toxin secreted by DAEC can change the paracellular permeability resulting in fluid accumulation in the intestine. Host interactions with Afa/Dr adhesins can induce migration of polymorphonuclear leukocytes (PMNLs) (3) which promotes the production of proinflammatory cytokines which in turn promote the upregulation of DAF (3). It was reported that finger-like projections are induced in enterocytes due to signal transduction caused by DAEC (4). The figure was created with Biorender.com.

2.4.4.2 Relationship with *Shigella*

In the past, enteroinvasive strains that cause shigellosis were divided into EIEC and *Shigella* species (with four subgroups *S. dysenteriae, S. flexneri, S. boydii, and S. sonnei*). Genetically all

these groups are so closely related they could be considered as the same genus or even species comprising other pathogenic and commensal *E. coli*. Like *Shigella*, EIEC strains are usually nonmotile, lysine decarboxylase negative, and commonly lactose negative (Belotserkovsky *et al.*, 2018; Echeverria *et al.*, 1991; Escobar-Páramo *et al.*, 2003). Biochemical similarity between EIEC and *Shigella* is characterized by their ability to utilize serine, xylose or sodium acetate and ferment mucate (Belotserkovsky *et al.*, 2018; Doyle, 1989). It has been reported that non-motile serotypes of EIEC produce an unusually large flagellin assembled into functional flagellum filaments (Andrade *et al.*, 2002). EIEC/Shigella utilizes similar modes of action, to manipulate host innate and adaptive immunity for penetration, multiplication, and replication in host epithelial cells (Belotserkovsky *et al.*, 2018; Kaper *et al.*, 2004; Kotloff *et al.*, 2018; Nasser *et al.*, 2022; Rojas-Lopez *et al.*, 2018; Schnupf *et al.*, 2019). Two main features of EIEC and *Shigella* are evolution requiring both the gain of virulence genes and the loss of function or deletion of other genes (Maurelli *et al.*, 1998). In *S. flexneri*, approximately 200 genes were acquired, and 900 genes have been lost during the divergence from commensal *E. coli* (Jin *et al.*, 2002; Wei *et al.*, 2003). Some of the examples include loss of outer membrane protease (OmpT) that interferes with the localization of the actin nucleator IcsA, which is necessary for the invasion phenotype; loss of lysine decarboxylase that catalyses cadaverine production, which inhibits enterotoxins of *Shigella* (Maurelli *et al.*, 1998; Sansonetti *et al.*, 1983); and the absence of functional flagella and fimbriae that are active activators of the host innate immune response, and might interfere with the initial colonization process (Bravo *et al.*, 2015; Ramos *et al.*, 2004)(other genomic regions “black holes” lost by EIEC and Shigella are reviewed in detail in (Belotserkovsky *et al.*, 2018)). Due to the similarities shared between EIEC and Shigella, virulence factors responsible for EIEC or *Shigella* infections and various subtypes of attenuated Shigella strains can be exploited to devise potential vaccine against these enteroinvasive pathotypes.

2.4.4.3 Vaccine strategies against EIEC/*Shigella*

EIEC/*Shigella* does not have any animal reservoir, they are usually transmitted among the human population through poor hygiene and sanitation. Licensed vaccines are currently not available against EIEC/*Shigella* and basic hygiene and proper sanitation remain the best way of preventing infection (MacLennan *et al.*, 2022; Mani *et al.*, 2016). However, these approaches are not feasible in many low-income countries. Therefore, there is an increased demand for vaccine development, and strategies include the utilization of a live-attenuated strain, glycoconjugate based candidates, novel antigen candidates such as *Shigella* outer membrane vesicles (OMV)

encapsulated in nanoparticles, and protein subunit candidates (Barel *et al.*, 2019; Camacho *et al.*, 2013a; Camacho *et al.*, 2013b; MacLennan *et al.*, 2022; Pastor *et al.*, 2018; Tian *et al.*, 2021). There are several animal and human studies which have described the potential for vaccination using invasion plasmid antigens (IPAs), *virG* or *Shigella* O antibodies that cross react with EIEC O antigens (Alexander *et al.*, 1996; Levine *et al.*, 2007). **table 2.4** summarizes vaccine approaches against EIEC/*Shigella*.

Table 2.4 Reports describing vaccines against Enteroinvasive *E. coli* (EIEC)/*Shigella*

Type of vaccine	Component of vaccine	Results/Observations/Outcomes/Animal model/Phase (Year)	References
Live-attenuated vaccine	ShigETEC, attenuated *Shigella* strain expressing ETEC antigens (LTB and detoxified version of ST)	Currently phase I clinical trials, yielded high titer IgG and IgA against bacterial lysates and anti-ETEC toxins (2022)	(Girardi *et al.*, 2022; Harutyunyan *et al.*, 2020)
	WRSs2, attenuated *ΔvirG S. sonnei* in which enterotoxin genes senA/senB are deleted.	Currently phase II clinical trials, NCT04242264 (2021)	(Venkatesan *et al.*, 2021)
	WRSs3, *attenuated ΔvirG S. sonnei* in which *senA/senB*, and acetyl transferase genes *msbB* are deleted.	Currently phase II clinical trials (2021)	(Venkatesan *et al.*, 2021)
	Shigella flexneri 2a, O antigen mutant (*Δwzy*) combined with *E. coli* LT mutant.	Provided cross-protective immunity against Shigella and ETEC. Pre-clinical stage (2018)	(Kim *et al.*, 2018)
	Formalin-inactivated trivalent *Shigella* whole cell vaccine	Phase II clinical trials (2022)	(MacLennan *et al.*, 2022)
Heat-killed multi serotype	Heat killed cocktail of 6 strains of *Shigella* inactivated vaccine	Pre-clinical stage (2016)	(Mani *et al.*, 2016)
Subunit vaccines	S4V-EPA, four-valent, O-antigen bioconjugates against *S. sonnei*, *flexneri 3a* and *flexneri 6*, and *S. flexneri 2a*.	Phase II clinical trials (2022)	(Martin *et al.*, 2022) Limmatech

	SF2a-TT15, *S. flexneri 2a* synthetic O-antigen conjugates against *S. flexneri 2a*	Phase II clinical trials (2022)	(Phalipon *et al.*, 2022)
	InvaplexAR-DETOX, artificially detoxified *S. flexneri 2a* invasin complex with recombinant IpaB/IpaC	Phase I clinical trial (2021)	WRAIR, (Venkatesan *et al.*, 2021)
	ZF0901, Bivalent O-antigen glycoconjugate against *S. flexneri 2a* and *S. sonnei*	Phase III clinical trial (2021)	Beijing Zhifei Lvzhu biopharmaceuticals, (Mo *et al.*, 2021)
	altSonflex 1-2-3, a four valent Shigella native outer membrane vesicles (OMVs) against *S. flexneri 1b, 2a, 3a,* and *S. sonnei*.	Phase II clinical trial (2022)	(MacLennan *et al.*, 2022)
Adjuvanted whole cell vaccine	Alum adjuvanted and CTB-adjuvanted EIEC whole-cell vaccine	Higher IgG yield and immune response against EIEC and ETEC in orally immunized mice (2016)	(Gohar *et al.*, 2016)

2.4.5 EAEC and DAEC

EAEC is the cause of several diarrheal outbreaks worldwide and is mostly associated with mildly inflammatory diarrhea in young (< 2 years old) and malnourished children; persistent diarrhea in HIV-infected adults and children and acute diarrhea in travellers in both developing and industrialized countries. It was first described in 1987 based on an auto-aggregative “stacked-brick” adherence pattern to Hep-2 cells in culture (Nataro *et al.*, 1987). This phenotype is mediated by the aggregative adherence fimbriae (AAF) and aggregative adherence regulator (Czeczulin *et al.*, 1997; Harrington *et al.*, 2006; Jenkins, 2018).

2.4.5.1 Molecular pathogenesis

EAEC infection involves colonization of bacterial cells in dense clusters to the intestinal mucosa (Czeczulin *et al.*, 1997; Nataro *et al.*, 1987) followed by secretion of EAEC heat-stable enterotoxin (EAST1) and ShET1 (*Shigella* enterotoxin 1) which cause loss of fluid (Fasano *et al.*, 1997; Savarino *et al.*, 1996) (**Figure 2.1**). EAEC also produces a plasmid-encoded SPATE autotransporter enterotoxin called Pet (Eslava *et al.*, 1998). Pet has enterotoxic activity and induces cytoskeletal changes and epithelial cell rounding due to the breakdown of fodrin/spectrin in host cells (Villaseca *et al.*, 2000). In addition, EAEC produces another SPATE with mucinase activity called Pic (Harrington *et al.*, 2009), which contributes to intestinal colonization. Pic was also shown to reduce complement activation by cleaving complement cascade factors - C3, C4 and C2 (Abreu *et al.*, 2015), also induces polymorphonuclear leucocyte/neutrophil (PMN) activation and programmed T-cell death (Ruiz-Perez *et al.*, 2011). In this context, Pic activity can contribute to immune evasion and promote EAEC virulence.

Like EAEC, the DAEC pathotype is defined by their diffused adherence pattern on epithelial cells such as HeLa or Hep-2 cells in culture. More than 70% of DAEC strains produce fimbrial adhesins from the Afa (AfaE-I, AfaE-II, AfaE-III and AfaE) or Dr (F185) family that mediate the diffused adherence phenotype and pathogenesis. These fimbriae recognise DAF (Delay accelerating factor), a cell-surface glycosyl phosphatidylinositol-anchored protein, as the receptor and cause dismantling of the actin network in intestinal cells, resulting in long cellular extensions and elongation and malfunction of microvilli which wrap around the bacteria (Le Bouguénec *et al.*, 2006; Meza-Segura *et al.*, 2020; Nataro *et al.*, 1987; Servin, 2005; Servin, 2014) (**3**
). Reports have suggested the presence of virulence factors other than Afa/Dr family adhesins and they can be pro-inflammatory suggesting a potential significance in the initiation of inflammatory bowel disease (Mirsepasi-Lauridsen *et al.*, 2019; Schultsz *et al.*, 1997). In addition to causing diarrhea in children, adults and the elderly (Canizalez-Roman *et al.*, 2016), DAEC are also known to be associated with urinary tract infections (UTIs), pregnancy complication and asymptomatic intestinal infection in both children and adults (Mohamed Ali, 2011; Servin, 2014).

2.4.5.2 Vaccine strategies against EAEC and DAEC

For DAEC and EAEC pathotypes, there is currently limited vaccine development. Nevertheless, bacterial adhesins are considered the best candidates, and AAF/I and AAF/II could be the possible target for EAEC. When Balb/c mice were immunized with three different modes of vaccination - DNA/DNA, DNA/Protein, or Protein/ Protein of AAF/I or AAF/II of EAEC, respectively,

only the DNA/Protein immunization and Protein/Protein doses of AAF/I significantly induced total IgG (Bouzari *et al.*, 2010).

Likewise, afimbrial adhesins (AFA), Dr hemagglutinin, and F1845 fimbriae are the important adhesins of DAEC and could be target antigens for vaccine development (Saeedi *et al.*, 2017). Vaccination of C3H/HeJ mice with *Escherichia coli* Dr fimbrial antigen reduced urinary tract infection due to a homologous strain bearing Dr adhesin. Immune sera with high titers of anti-Dr antibody inhibited bacterial binding to bladders and kidneys (Goluszko *et al.*, 2005). Although Dr adhesin was shown to be immunogenic in this urinary tract model, its role as an antigen in vaccination against intestinal disease is yet to be explored.

2.4.6 AIEC

In contrast to enteroinvasive *E. coli*, Adherent-invasive *E. coli* (AIEC) is the pathotype with both abilities to adhere to and invade intestinal epithelial cells. AIEC can also survive and replicate within macrophages (Kaper *et al.*, 2004; Smith *et al.*, 2013). AIEC is associated with inflammatory bowel disease (IBD), Crohn`s disease (CD) and ulcerative colitis (UC) (Chervy *et al.*, 2020; Conte *et al.*, 2014; Martinez-Medina *et al.*, 2009; Palmela *et al.*, 2018). AIEC infections are usually characterised by diarrhea, abdominal pain, rectal bleeding, fatigue, and life-threatening complications in severe cases of AIEC and IBD. Reports of Bacteriological analysis from Europe and North America showed 30-50% of Invasive *E. coli* strains are present in the ileal mucosa of Crohn's disease patient (Darfeuille-Michaud *et al.*, 2004; Denizot *et al.*, 2012; Kamali Dolatabadi *et al.*, 2021; Leccese *et al.*, 2020). However, it is still not clear whether AIEC induces intestinal inflammation resulting in IBD or whether they act as an intensifying factor by colonising the mucosa of patients with pre-existing IBD. AIEC possesses a wide range of virulence factors.

2.4.6.1 Molecular pathogenesis

AIEC invade host cells in a similar fashion to other pathogens like EIEC and *Shigella*. These strains penetrate epithelial cells by either directly invading the epithelial layer or by entering via microfold (M) cells, in the epithelium of the small intestine. Following invasion, AIEC reduce epithelial barrier function and integrity (Smith *et al.*, 2013). Since CD and AIEC are interrelated, most of the studies of AIEC pathogenesis were done in epithelial cells from CD patients. During initial adherence, it has been shown that type 1 fimbriae can bind to GP2 protein located on the apical plasma membrane of M cells (Bringer *et al.*, 2015; Smith *et al.*, 2013). Reports also suggest that AIEC strains expressing long polar fimbriae (LPF) adhere to M cells and mediate transcytosis of

bacteria. Host glycoprotein CEACAM6 (Carcinoembryonic Cell Adhesion Molecule 6) expressed in ileal mucosa has been shown to recognize the FimH type 1 fimbrial adhesin and facilitate internalization of AIEC (Barnich *et al.*, 2007). Outer membrane vesicles (OMVs) have role in AIEC invasion. It has been demonstrated that outer membrane proteins (OmpA amd OmpC) promote fusion of OMVs with the Gp96 receptor expressed on the surface of epithelial cells and mediate invasion (Rolhion *et al.*, 2010; Rolhion *et al.*, 2011). In addition, VAT-AIEC a protease secreted by AIEC has been reported to mediate colonization of bacteria in a murine intestinal infection model (Gibold *et al.*, 2016). Following invasion, AIEC strains can replicate in host cells including macrophages (Prudent *et al.*, 2021).

2.4.6.2 Relation of AIEC and Crohn's disease

Crohn's disease (CD) is a chronic inflammatory condition of the GI tract and AIEC strains have been isolated from guts of CD and UC patients (Conte *et al.*, 2014; Palmela *et al.*, 2018). AIEC strains are pathobionts, which means they can reside in the guts of healthy individuals without causing any diseases. However, the host intestinal environment under certain conditions that affect the innate immune response could promote AIEC induced inflammatory bowel diseases (Chassaing *et al.*, 2014; Nadalian *et al.*, 2021). Following AIEC infection, autophagy which is a cytosolic process is induced in host cells to curb the activity of bacteria, occurs. During autophagy, bacteria are recognised by NOC protein such as CD-associated nucleotide-binding oligomerization domain-containing-2 (NOD2) and form autophagosomes which further fuse with lysosomes. After fusion the bacteria are degraded in auto-phagolysosomes (Negroni *et al.*, 2016). In addition to NOC proteins, other proteins such as ATG16L1, IGRM, and LC3 are also known to mediate autophagy (Palmela *et al.*, 2018; Smith *et al.*, 2013). It has been reported that these mediators of autophagy are defective in CD and UC patients and may favour intra-macrophagic replication of AIEC. Genetics models have reported that knock-down of NOC proteins show a loss of function against AIEC infections. Knock-down of *ATG16L1* and *IRGM* genes result in defective clearance of CD associated AIEC (Denizot *et al.*, 2012; Homer *et al.*, 2010).

2.4.6.3 Insights for vaccine development

In recent years, many studies have elucidated AIEC virulence and their role in CD. Various therapeutic strategies other than vaccination to prevent AIEC infection and concurrent CD and UC have been tested. Among these, most popular strategies are i) use of prebiotics/probiotics and postbiotics and chemical compounds to inhibition or exclude AIEC. ii) Elimination of AIEC using

antibiotics, bacteriocins/colicins, phototherapy, and bacterial predation, iii) activation of autophagy, iv) Nutritional interventions, v) fecal transplantation and combinational therapy (Alvarez Dorta *et al.*, 2016; Boudeau *et al.*, 2003; Chalopin *et al.*, 2016; Sivignon *et al.*, 2015; Van den Abbeele *et al.*, 2016; Yan *et al.*, 2015). One vaccine-based approach involves use of flagellar antigens. A Summary of approaches for AIEC and IBD prevention are listed in **table 2.5**.

Table 2.5 Reports on vaccines other strategies against Adherent invasive *E. coli* (AIEC) and Inflammatory bowel disease (IBD)

Type of vaccine	Component of vaccine	Results/Observations/Outcomes	Animal model (Year)	References
Adjuvanted enhanced vaccine	Intranasal immunization of mice using siderophore enterotoxin (Ent) conjugated with CTB.	Increased fecal antibodies against Ent and reduced AIEC colonization in immunized mice.	Orally immunized mice (2021)	(Hossain, 2021)
	CTB-Ent, immunization of mice.	Mucosal IgA against Ent and GlcEnt, protection from systemic infection and decreased AIEC and Crohn's disease and colitis in mice.	Orally immunized mice (2022)	(Gerner *et al.*, 2022)
Inhibition of FimH adhesin	Thiazolylaminomannosides and n-heptyl α-D-mannose based inhibition of AIEC LF82 adherence to colon tissue by blocking FimH	FimH blocker molecule EB8018/TAK-018 is under phase 2a clinical trial (NCT03943446)	Clinical trial (2020)	(Chervy *et al.*, 2020)
Probiotics, prebiotics, and postbiotics	Probiotics containing portion of *S. cerevisiae* CNCM I-3856	Known to prevent colitis induced by AIEC in mouse model of Crohn's disease.	Orally immunized mice (2018)	(Roussel *et al.*, 2018)
	Probiotics *Lactobacillus*	Known to reduce AIEC survival and growth.	Orally immunized mice (2018)	(Mantegazza *et al.*, 2018)

	rhamnosus GG and *Lactobacillus reuteri.*			
	Prebiotics containing long chain arabinoxylans	Known to inhibit mucin adhesion of AIEC.	Orally immunized mice (2020)	(Nguyen *et al.*, 2020)
	Prebiotics containing insulin and galacto-oligosaccharides	Limit AIEC survival and growth	Orally immunized mice (2017)	(Canfora *et al.*, 2017)
	Postbiotics like colicins E1 and E9 that are species-specific bacteriocins.	Known to kill intracellular, biofilm forming and cell adhering AIEC.	Mice model (2018)	(Kittana, 2018)
Fecal microbiota transplantation (FMT)	Restoration of normal intestinal flora to prevent CD and AIEC colonization	Placebo-controlled trials using FMT has shown improvements in patients with active disease.	Pre-clinical trial stage (2022)	(Pickard *et al.*, 2017)
Phage Therapy	LF82 bacteriophages that were able to replicate in ileal, colon samples and feces.	Oral dose of bacteriophages has inhibited AIEC strain LF82 colonization and colitis symptoms in gut. Phase 1/2a clinical trial ongoing.	Human volunteers (2015)	(Zhang *et al.*, 2015)Mount Sinai hospital
	Five-phage cocktail against IBD	IBD suppression. Currently in Phase II clinical trial (NCT04737876).	Clinical trial (2022)	(Kotsiliti, 2022)

2.5 Extraintestinal Pathogenic *E. coli* (ExPEC)

Extraintestinal Pathogenic *E. coli* (ExPEC) are responsible for most urinary tract infections (UTIs), are a common cause of blood stream infections and neonatal meningitis in humans and respiratory tract and other systemic infections in poultry. This group of pathogens have a broad range of virulent factors and exhibit genomic plasticity. Depending on the type of infectious disease, ExPEC have been classed into uropathogenic *E. coli* (UPEC), neonatal meningitis-associated *E. coli* (NMEC), and sepsis-causing *E. coli* (SEPEC) in humans and avian pathogenic *E. coli* (APEC)

in poultry. Some ExPEC that cause infections in humans share close phylogenetic relationship and virulence genes with certain APEC strains. Studies have shown that poultry and poultry products can be a reservoir of potential ExPEC strains and could pose a health risk to humans. Both animal and human ExPEC strains were shown to be able to cause UTI or meningitis in rodent models; and human ExPEC were shown to be virulent in poultry infections as well (Bauchart *et al.*, 2010; Moulin-Schouleur *et al.*, 2007; Skyberg *et al.*, 2006; Tivendale *et al.*, 2010). As such, some ExPEC strains have clearly been shown to infect multiple host species (Habouria *et al.*, 2019).

2.5.1 Uropathogenic *E. coli* (UPEC)

Urinary tract infection is a very common bacterial infection, particularly in women, and UPEC strains account for up to 90% of community acquired urinary tract infections (UTIs), 50% of hospital acquired UTIs, and around 80% of uncomplicated (Foxman, 2010; Tabasi *et al.*, 2016). UPEC isolates exhibit a high degree of genetic diversity due to the presence of mobile DNA segments which are scattered around the chromosome known as pathogenicity islands. The key virulence factors harbored by UPEC strains are type 1 fimbriae, P fimbriae, mannose-resistant adhesins, hemolysin, serum resistance, siderophores and K1 capsule (Kaper *et al.*, 2004; Lloyd *et al.*, 2007). Normally, UTI infection is initiated when uropathogenic *E. coli* originating from the bowel, is transferred to the urogenital region and ascends towards the periurethral area. Colonization is facilitated by adhesins like Pap (P), type 1 and other fimbriae like S, M, and F1C that mediate adherence to uroepithelial cells, which is an important initial step in the development of UTI. It has been shown that by virtue of type 1 fimbriae, *E. coli* can attach to mannose moieties that coat transitional epithelial cells (Dhakal *et al.*, 2012; Navarro-Garcia *et al.*, 2010). Further, several toxins including hemolysin, a serine-protease autotransporters called Vat, Sat and Pic and the cytotoxic necrotizing factor (CNF-1) can contribute to urinary tract colonization. However, these virulence factors are not always present among different subgroups of UPEC, suggesting that there can be multiple mechanisms of UPEC pathogenesis (Zhao *et al.*, 2009).

2.5.1.1 Urinary Tract Infection (UTI)

The urinary tract can be affected by a variety of diseases including microbial colonization of the urine and infection of the urinary tract tissues - kidney, renal pelvis, ureters, bladder, and urethra. Clinically, urinary tract infection (UTI) has been categorized as uncomplicated or complicated. Uncomplicated UTIs occur in the normal urinary tract of immunocompetent individuals who are otherwise healthy (Terlizzi *et al.*, 2017). UTI is characterized by the presence of bacteria and

neutrophils in the urine which is known as bacteriuria (presence of uropathogens in urine with more than 10^5 CFU/ml) (Schmiemann *et al.*, 2010). Bacteriuria can be asymptomatic in the host and have adverse outcomes for pregnant women and people undergoing traumatic genitourinary procedures (Ranjan *et al.*, 2017). UTI can manifest itself into cystitis (inflammation of the bladder) and acute pyelonephritis (kidney infection) (**Figure 2.3**). Cystitis is typically characterized by symptoms including frequency, burning sensation, urgency, pyuria (leukocytes in urine), dysuria, suprapubic pain and/or lower abdominal discomfort with cloudy urine. If left untreated, these infections can result in pyelonephritis which is distinguished clinically from cystitis by the presence of flank pain, fever, and nausea (Wagenlehner *et al.*, 2015). Antibiotics are given for the treatment of symptomatic UTI but up to 25% will suffer a recurrence of infection within 6 months following treatment of initial UTI (Foxman, 2014). Mounting evidence is showing that two-thirds of these recurrences are attributable to the identical initial strain recovered from a given patient suffering from uncomplicated UTIs (Ejrnæs, 2011; Ikähelmo *et al.*, 1996; Nielsen *et al.*, 2021).

2.5.1.2 Molecular pathogenesis of UPEC

The pathogenesis of UTI is complex and influenced by multiple host and microbial factors. Years of research and use of different animal and cellular models have elucidated some of the pathogenic mechanisms. The mechanisms of UPEC infection include adherence to host cells, motility, acquisition of essential metals and other micronutrients, toxin production and evasion of the host immune response (**Figure 2.3**).

Bacterial adherence to host cells plays a key initial step in colonization and subsequent disease progression. Uropathogens must adhere to or penetrate the mucosal barrier to persist. A UTI typically initiates by contamination of the periurethra by a uropathogen from intestinal sources, followed by bacterial colonization of the urethra and bladder through filamentous adhesins known as fimbriae (pili) (O'Connell Motherway *et al.*, 2019). For example, UPEC strain CFT073, a well-characterized reference strain, encodes 12 distinct fimbrial gene clusters which code for type 1, P, F1C, Dr, Auf fimbriae as well as their chaperone and usher proteins (Welch *et al.*, 2002). Type 1 fimbriae have been found essential for colonization, invasion, and persistence of UPEC in the mouse bladder. The FimH adhesin of type 1 fimbriae binds to mannosylated receptors and bladder cell surface known molecules such as uroplakins, α 3 β 1 integrins and the pattern recognition receptor TLR4 (Ashkar *et al.*, 2008; Kisiela *et al.*, 2015; Zhou *et al.*, 2001). The expression of type 1 fimbriae is phase variable and controlled by the orientation of an invertible element in the promoter region (Abraham *et al.*, 1985; Bjarke Olsen *et al.*, 1994).

E. coli expresses another fimbria known as P fimbriae which have been associated with acute pyelonephritis in humans and showed a subtle role for pathogenesis in the murine model (Lane *et al.*, 2007; Wullt *et al.*, 2000). P fimbrial adherence conferred by the PapG adhesin protein binds specifically to glycosphingolipids containing digalactoside moieties found in renal epithelium and the P blood group antigen which is on the surface of some host erythrocytes (Lund *et al.*, 1987). So, the human individuals lacking the receptor for P fimbriae may be less susceptible to P-fimbriae-mediated adherence during UTIs caused by UPEC.

Type 1 fimbriae bind to epithelial cells and trigger a signal transduction cascade that activates the Rho family of GTP binding proteins, resulting in cytoskeleton rearrangements in host cells and internalization of UPEC by a zippering mechanism in which the plasma membrane engulfs the bacterium (Martinez *et al.*, 2000). This invasion benefits bacteria since the intracellular location shelters UPEC from host defenses, may reduce access to antibiotics, and prevent clearance from micturition. Although intracellular UPEC can evade host defenses, expulsion of UPEC in urothelial cells does occur by innate immune defense through lipopolysaccharide (LPS) mediated activation of TLR4 (Song *et al.*, 2009). However, a minority of internalized UPEC can escape into the epithelial cell cytoplasm and subvert expulsion and rapidly replicate exponentially in coccoid form, forming an amorphous biofilm-like intracellular bacterial community (IBC) which can cause superficial cells to protrude (Justice *et al.*, 2004). Later, maturation of IBCs can lead to bacterial dispersion and the cycle of invasion of other urothelial cells (Justice *et al.*, 2004). Alternatively, UPEC can establish quiescent intracellular reservoir (QIRs) in the underlying bladder cells which can later serve as seeds for UPEC release back into the bladder lumen (Mysorekar *et al.*, 2006; Schilling *et al.*, 2001). Due to this phenomenon, the source of recurrent infections can be due to several sources including recurrent intestinal source contamination, vaginal colonization, or reinfection by latent bacteria within the urinary tract.

Other virulence determinants of some UPEC include hemolysin, cytotoxic necrotizing factor 1 (CNF1) and autotransporter proteins. Hemolysin (HlyA), encoded by the *hlyCABD* operon, oligomerizes and inserts into the host cell membrane in a Ca^{+2} dependent manner (Wiles *et al.*, 2008). Hemolysin is implicated in pore formation in bladder cells and promotes their lysis and facilitates iron and nutrient acquisition (Dhakal *et al.*, 2012). Further, it can trigger cell exfoliation, apoptosis, and cytokine production and induce an inflammatory response (Bien *et al.*, 2012; Dhakal *et al.*, 2012). CNF1 secreted by some UPEC strains alters actin remodeling and membrane ruffling, which leads to the internalization of UPEC in the host cell through the activation of Rho GTP-binding proteins: Rac1, RhoA and CDC42 (Visvikis *et al.*, 2011) and may also play a role in bladder cell exfoliation (Mills *et al.*, 2000). In addition, activation of Rac1 and GTP induces the anti-apoptotic

pathway, preventing apoptosis of the colonized uroepithelium and prolonging UPEC survival (Miraglia *et al.*, 2007). Another family of toxins – members of the serine protease autotransporters of *Enterobacteriaceae* (SPATEs) including Sat, Pic and Vat have been characterized in UPEC. Sat (secreted autotransporter protein) can cause cytotoxic effects on bladder and kidney cells *in vitro* (Guyer *et al.*, 2000); elongation of kidney cells with apparent impairment of cellular junctions (Guyer *et al.*, 2002); degradation of fodrin and human coagulation factor V (Dutta *et al.*, 2002); and induction of autophagic cell detachment (Liévin-Le Moal *et al.*, 2011). The Pic SPATE acts as a mucinase (Henderson *et al.*, 1999) and Vat SPATE can also contribute to UTIs (Díaz *et al.*, 2020; Nichols *et al.*, 2016).

To survive and compete in different host niches, UPEC also evolved multiple means of obtaining essential metals, such as iron. The most diverse and broadly distributed iron uptake mechanism used by microorganisms are siderophore acquisition systems. Siderophores are small chelating compounds with a very high affinity for iron (Jones *et al.*, 1980). The direct capture of host iron is either from free heme or from heme-containing proteins, such as hemoglobin. The presence of heme uptake systems in UPEC also provides direct iron source *in vivo,* heme. Iron binding receptors Hma and ChuA bind to heme and transport it to the periplasm; ChuT mediates further transfer to the cytoplasm through an ATP-binding cassette (ABC) transporter (Hagan *et al.*, 2009; Stojiljkovic *et al.*, 2002). In addition, UPEC can sequester host iron through high-affinity siderophores including salmochelins, C-glycosylated derivatives of enterobactin, and other siderophores, such as aerobactin, and yersiniabactin (Henderson *et al.*, 2009). Salmochelins are encoded by the *iroBCDEN* gene cluster. This siderophore contributes to the virulence of ExPEC strains by escaping the action of host lipocalin-2 (siderocalin) (Raffatellu *et al.*, 2009). The aerobactin siderophore is highly expressed and stable and displays a higher affinity than enterobactin at low pH (Valdebenito *et al.*, 2006). Interestingly, yersiniabactin which is a confirmed siderophore, also contributes to UPEC resistance to copper toxicity in urine (Chaturvedi *et al.*, 2012). Like iron, zinc is also an essential element for bacterial growth. The high-affinity zinc transport system ZnuACB has been expressed during UTI to acquire zinc in the host (Sabri *et al.*, 2009).

Polysaccharidic capsules and serum resistance are also important traits associated with UPEC strains especially those which can infect the kidney and develop into systemic infections (Cross *et al.*, 1986). Antiphagocytic capsules can reduce the uptake of bacteria by host phagocytes and promote dissemination in blood and extra-intestinal tissues.

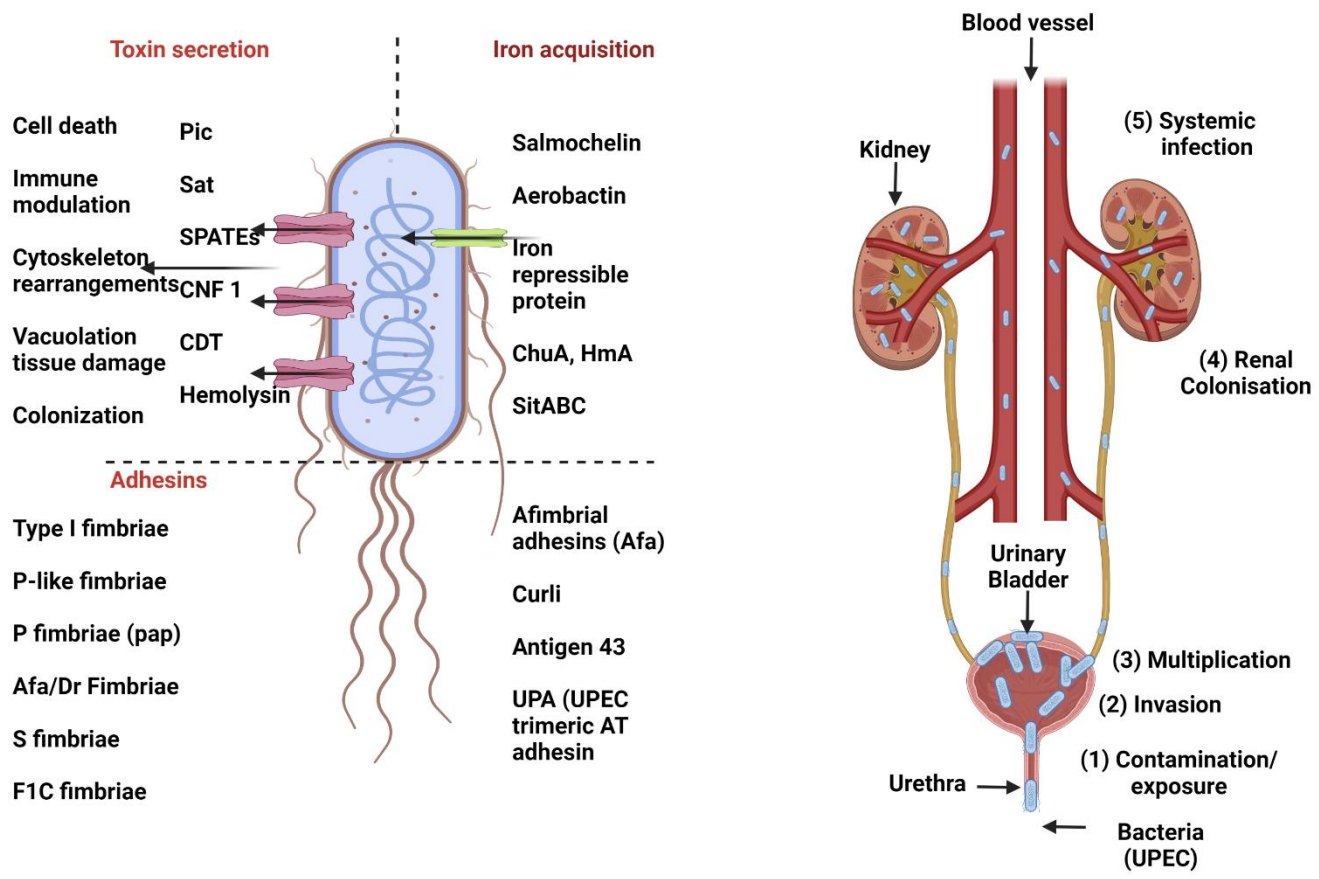

Figure 2.3 Virulence factors and pathogenesis of UPEC.

UPEC possess an array of virulence factors that are distinct from those found in the intestinal pathotypes and hence allow them to colonize extraintestinal sites. Adherence is the first stage of colonization mediated by adhesins such as Type 1 fimbriae, P fimbriae, curli, and outer membrane adhesins. Each adhesin has a specific host receptor for example type 1 fimbriae recognize manno-oligosaccharides present on glycoprotein molecules, P fimbriae recognize the digalactoside component of P blood group antigen present in uroepithelial cells. UPEC produce other adhesins like Dr adhesins, F1C fimbriae and S fimbriae. Type 3 and F9 fimbriae have been associated with the formation of biofilm and catheter associated UTIs. Iron is scare inside the host, but UPEC can glean host iron through high-affinity siderophores including salmochelin, C-glycosylated derivatives of enterobactin, and the hydroxamate siderophore, aerobactin. The presence of heme uptake systems in UPEC help them to acquire a readily available iron source *in vivo*, heme. Iron binding receptors Hma and ChuA bind to heme and the coordinated molecule is transported into the periplasm; ChuT mediates further transfer to the cytoplasm through an ATP-binding cassette (ABC) transporter. UPEC can also produce hemolysin (HlyA), cytotoxic necrotizing factor 1 (CNF 1) and autotransporter proteases like Sat, Tsh, Vat, TagBC, Sha. In ascending urinary tract infection (UTI), bacteria colonize the urethra and ascend to the bladder leading to cystitis (1) and sometimes subsequently the kidneys resulting in pyelonephritis (4). Bacteria adhere to the bladder surface via type 1 fimbriae

which can also mediate cell invasion (2) and replicate (3) within the cell cytoplasm. When the bacteria reach the kidneys (4) there is increased risk for septicemia (5). The figure was created with BioRender.com.

2.5.1.3 Vaccine strategies against UPEC

Generally, antibiotics are used to treat UTIs. However, increased antibiotic resistance has become an obstacle for UTI treatment (O'brien *et al.*, 2017). Recently World Health Organization (WHO) revealed that UPEC strains causing UTI are 54.4% resistant to first-line antibiotics co-trimoxazole and 43.1% resistant to broad-spectrum ciprofloxacin (Kot, 2019; Mortazavi-Tabatabaei *et al.*, 2019; Organization, 2020). Fluoroquinolone resistant *E. coli* are widespread in the world and there are countries where Fluoroquinolone treatment is completely ineffective in more than 50% of patients (Aarestrup *et al.*, 2003). To address such issues vaccination against UTI strains might provide an important alternative to antibiotics for UTI prevention, particularly in individuals more susceptible to recurring infections.

An effective vaccine against UPEC might help reduce current spread of infection and morbidity rate of patients as well as address the challenge of treatment of some UTIs. Effective UPEC vaccine strategies should consider the following aspects: i) Heterogeneity of UPEC strains. ii) The potential side effects on the commensal microbiota of the intestine, and iii) Production of multiple virulence factors by UPEC strains. Since UPEC infections involve multiple steps (**Figure 2.3**), an effective vaccine should be able to provide a protective immune response against key virulence factors required at specific stages of UTI pathogenesis, such as colonization, invasion, and the formation of IBC reservoirs. Currently vaccines against UPEC are being developed using either cell-based or live-attenuated vaccines, killed bacteria, or antigen-based subunit vaccines against antigens such as, toxins and polysachharide based conjugate vaccines (Karam *et al.*, 2019).

Solco-Urovac is a whole bacterial cell-lysate based vaccine that is multivalent and comprises cells for 10 distinct bacterial strains (6 UPEC strains, 1 strain of each *Proteus mirabilis*, *Klebsiella pneumoniae*, *Morganella morganii* and *Enterococcus faecalis*). This vaccine is known to protect against recurrent UTIs, and multiple doses reduce the prevalence of cases caused by UPEC (Hickling *et al.*, 2013; Kochiashvili *et al.*, 2014; Prát *et al.*, 1989). However, this vaccine caused major side effects such as fever, burning, bleeding, vaginal itching and nausea (Hopkins *et al.*, 2007). Such deleterious side effects dismiss the use of Urovac as a vaccine against UTIs. Uro-vaxom, is an oral vaccine against UTIs, which was commercialized in Switzerland in 1994, and later in other countries (Cruz *et al.*, 2009; Trial, 1994; Wade *et al.*, 2019). This vaccine comprises membrane proteins of 18 UPEC strains and is known to effectively prevent recurrent UTIs in women. Uro-vaxom has significantly less side effects than Urovac, however repeated doses taken every 3 months are

required, which has raised issues concerning the practicality of using this vaccine in some population of patients (Kim *et al.*, 2010a). Other vaccines against UTIs include Urvakol and Urostim that each contain a mixture of killed bacterial pathogens including *E. coli*, *P. mirabilis*, *E. faecalis*, and *K. pneumoniae* (Urostim) and an additional *Pseudomonas aeruginosa* strain (Uravakol). These two vaccines promote immunogenicity in animals and humans, but clinical trials for Uravakol and Urostim have not been completed (Magistro *et al.*, 2019; Marinova *et al.*, 2005). ExPEC9V is a newer vaccine which is currently in phase 3 trial (NCT04899336) and targets Invasive Extraintestinal Pathogenic *Escherichia coli* Disease (IED) in individuals aged 60 years and older with history of UTIs (Del Bino *et al.*, 2022). In addition to this, several attempts were also made to design live-attenuated vaccines by mutating capsular or somatic O antigens of UPEC strains. For example, attenuated strains such as CP923 and NU14 Δ*waaI* have been shown to produce significant humoral immune responses in mice (Billips *et al.*, 2009; Russo *et al.*, 2007). However, protection from UTI has not been demonstrated yet and optimization and continued testing of such vaccine candidates is ongoing (Forsyth *et al.*, 2020; Van den Dobbelsteen *et al.*, 2016).

As other strategies, antigen-based vaccines such as capsular- or LPS-based vaccines, fimbrial and non-fimbrial adhesin vaccines, iron scavenger receptor-based, and toxin-based vaccines have been investigated as protective targets (Huttner *et al.*, 2017; O'brien *et al.*, 2017). Currently in phase 2 clinical trial, ExPEC4V (NCT03500679), is a polysaccharide-based vaccine which contains O-antigens specific to serogroups O1A, O2, O6A and O25B. Two doses of this vaccine can prevent UTI even with higher bacterial doses and decrease bacteremia in women aged 18 years or older (Frenck Jr *et al.*, 2019; Smith *et al.*, 2019). UPEC colonization to the bladder epithelium is promoted by adhesins which also trigger host immune responses. This principle can be utilized to develop anti-adhesin based vaccines against UPEC. The FimH adhesin from type 1 fimbriae plays an important role in UPEC pathogenesis. It has been shown that a vaccine comprised of truncated FimH or a complex of FimH with chaperone FimC (FimCH) were able to prevent colonization of different UPEC strains in a murine model. In addition, the FimCH vaccine with Freund's adjuvant and the FimH vaccine with Alum and MF59 adjuvants promoted an immune responses against UPEC and prevented colonization in murine and primate models respectively (Hasanzadeh *et al.*, 2020; Karam *et al.*, 2013a). The FimH vaccine was tested in a phase II clinical trial, however it was later rejected due to ineffectiveness in humans. Because of variation of expression of type 1 fimbriae, UPEC strains are not consistently recognized by the immune system (Karam *et al.*, 2019). In addition, antigens developed against the fimbriae do not target its mannose-binding region, and differences in the expression of virulence genes in animal and human models are some of the reasons for the failure of the FimH based vaccine. An effective UTI vaccine will

potentially require multiple tests using a variety of target antigens in urinary tract models in animals (Karam *et al.*, 2019; O'brien *et al.*, 2017). Other vaccine candidates using FimH adhesin are currently being tested including a TLR ligand-based vaccine where fusion of FimH adhesin to flagellin of UPEC as a TLR5 ligand was able to induce immune response and protected mice against UTI (Karam *et al.*, 2016; Karam *et al.*, 2013b; Savar *et al.*, 2014). Moreover, a PapG fimbrial adhesin based vaccine and Dr fimbriae with Freund's adjuvant were also shown to generate immune responses and reduce UPEC colonization in a mouse model (Spaulding *et al.*, 2016).

E. coli iron acquisition systems also have potential as UPEC vaccine candidates. It has been reported that the salmochelin receptor IroN or IroN with Freund's adjuvant can provide protection against UTI in a murine model (Russo *et al.*, 2003). The aerobactin receptor IutA conjugated with cholera toxin and the yersiniabactin receptor FyuA with Alum as adjuvant also generated strong immune responses and protection against UPEC infection in murine models (Brumbaugh *et al.*, 2013; Habibi *et al.*, 2017). In another study, the efficacy of a multi-epitope vaccine composed of siderophore receptors had been evaluated using an attenuated *Salmonella* vaccine delivery system, where IroN, IutA, IreA and FyuA improved protection in the urinary tract of mice (Wieser *et al.*, 2012; Wieser *et al.*, 2010). In the same line, a toxin-based vaccine containing antigens such as hemolysin HlyA, recombinant hemolysin, or mutated CNF1 and HlyA toxins, have reduced UTI symptoms in murine experimental infection models (Karam *et al.*, 2019; O'brien *et al.*, 2017). Auto-transporter toxins such as Vat from UPEC strains have also been investigated as vaccine targets. In the sepsis model study, Vat improved protection by 32% and 78% after active and passive immunization, respectively, compared to unvaccinated controls (Durant *et al.*, 2007).

2.5.2 MNEC, SEPEC

This ExPEC pathotype is associated with cases of meningitis particularly in neonates (Dietzman *et al.*, 1974). Approximately 80% of isolates from neonatal meningitis harbor antiphagocytic K1 capsular polysaccharide (Kim *et al.*, 1992; Robbins *et al.*, 1974) and most belong to serogroups such as O18, O7, O16, O1, and O45 (Bonacorsi *et al.*, 2003). Inflammation of the meninges by MNEC comprises various stages of infection – firstly, the translocation of bacteria into the circulatory system and upon reaching at least 10^3 CFU/ml in the blood, MNEC can breach the blood-brain barrier (BBB). The meninges which form a structural and functional blood-brain barrier are composed of endothelial cells called brain microvascular endothelial cells (BMECs). Most MNEC produce S fimbrial adhesins involved in BMEC binding to the NeuAc α-2,3-galactose receptor (Prasadarao *et al.*, 1996), type 1 fimbriae, and OmpA (Teng *et al.*, 2005), that contribute to

adherence and invasion of brain endothelial cells. Furthermore, an outer membrane protein, NlpI, can also mediate binding/invasion of MNEC to brain endothelial cells (Teng *et al.*, 2010). The CNF1 toxin through activation of the RhoA pathway may also contribute to MNEC invasion of BMECs and penetration into the brain (Khan *et al.*, 2002). IbeA has also been associated with the invasion of BMEC (Huang *et al.*, 2001). In addition to the role of IbeA in invasion and crossing of BBB, it has been linked to regulation of expression of type 1 fimbriae (Cortes *et al.*, 2008) which can mediate endothelial cell adherence and invasion. Further, IbeA may contribute to oxidative stress resistance (Fléchard *et al.*, 2012) and protection of bacteria against the H_2O_2 stress. The K1 polysaccharide capsule can contribute to serum resistance, antiphagocytic properties and intracellular survival (Cross *et al.*, 1986) and as such, it is an important virulence factor of most MNEC strains.

2.5.2.1 Vaccine strategies against MNEC

Despite much effort, no vaccine has yet been developed to help prevent neonatal meningitis caused by *E. coli*. The vaccine development is impeded by technical difficulties. There are well established animal models of MNEC induced meningitis in neonatal mice and rats to investigate MNEC pathogenesis (Kim, 2012; Wang *et al.*, 2021; Zhu *et al.*, 2020), but vaccine assessment is not favorable because it takes 2-3 weeks for active immunization (Nguyen *et al.*, 2016). Since this is a devastating disease among newborns and premature infants, alternative prophylactic therapies such as passive protection antibodies can be beneficial for the control of MNEC infection (Kim *et al.*, 1985; Zhu *et al.*, 2020). There has been research on capsular polysaccharides (CPs) as a good candidate for vaccine development but O-acetylated colominic acid produced by *E. coli* K1 was poorly immunogenic due to its similarity to polysaccharides found on the surface of human tissues (Finne *et al.*, 1983). Preventative approaches against MNEC may benefit by using virulence proteins as vaccine targets or for generation of protective antibodies for passive immunotherapy of neonates against MNEC (Rodrigues *et al.*, 2018).

2.6 Pathogenic *E. coli* of Importance to Animal Health

The first section of this review is focused on *E. coli* pathotypes associated with human infections and vaccination strategies to potentially prevent pathogenic *E. coli* from causing disease in humans. Importantly, *E. coli* is also an important pathogen of livestock and poultry and can cause both enteric and systemic infections. As such, it is important to consider the pathotypes of *E. coli* associated with infections of importance to animal health and potential approaches to prevent such infections through vaccination strategies.

2.6.1 Avian Pathogenic *Escherichia coli* (APEC)

Avian colibacillosis is a common disease in the poultry industry worldwide. As such, APEC is a major cause of morbidity and mortality in chickens due to localized or systemic infections, resulting in heavy economic losses to the poultry industry (Saif, 2009). APEC also infects other avian species including turkeys and ducks (Emery *et al.*, 1992; Olsen *et al.*, 2011; Wei *et al.*, 2013). A variety of disease types have been observed: including yolk sac infection, omphalitis, swollen head syndrome, respiratory tract infection, septicemia, enteritis, and cellulitis. Colibacillosis in chickens results in death by septicemia in acute infection, whereas subacute infections can result in pericarditis, airsacculitis and perihepatitis (Norton *et al.*, 2000). APEC strains commonly belong to three serogroups, O1, O2, and O78 although many different serogroups have been identified in APEC and O1, O2, and O78 are not always predominant in certain studies (Ewers *et al.*, 2004). Several virulence factors have been associated with APEC strains (Feng *et al.*, 2023). These include fimbrial adhesins (type 1 fimbriae (La Ragione *et al.*, 2000), P fimbriae (Johnson *et al.*, 2009), and Stg fimbriae (Lymberopoulos *et al.*, 2006), curli (La Ragione *et al.*, 2000), Yqi (Aleksandrowicz *et al.*, 2021); outer membrane proteins and other surface molecules contributing to serum resistance or antiphagocytic properties (Increased serum survival (Iss) (Foley *et al.*, 2000; Nolan *et al.*, 2003b), two-component signal transduction systems (RstA/RstB (Gao *et al.*, 2015b), PhoB/PhoR (Chakraborty *et al.*, 2011), BarA/UvrY (Herren *et al.*, 2006), O78 lipopolysaccharide, and K1 capsule (Mellata *et al.*, 2010; Mellata *et al.*, 2003); iron and metal acquisition systems (aerobactin (Gao *et al.*, 2015a), salmochelin (Caza *et al.*, 2008), yersiniabactin (Perry *et al.*, 2011)), heme utilization/transport protein ChuA (Gao *et al.*, 2012) and the Sit metal transport system (Sabri *et al.*, 2008)); autotransporters (Tsh, Vat, Sha, TagB, TagC AatA (Dozois *et al.*, 2000; Li *et al.*, 2010; Parreira *et al.*, 2003; Pokharel *et al.*, 2020), phosphate transport and the Pho regulon (the Pst system) (Lamarche *et al.*, 2005); sugar metabolism (Chouikha *et al.*, 2006); and nitrite transporter NirC (de Paiva *et al.*, 2015). Specific virulence genes including *iss*, *iroN*, *ompT, iutA* and *hlyF* are commonly present in APEC and are frequently encoded on large plasmids such as Colicin V (ColV) plasmids (Johnson *et al.*, 2006). In addition, other factors such as IbeA, and the type VI secretion system, which are known to affect expression of type I fimbriae, are involved in colonization of the respiratory tract (Germon *et al.*, 2005). However, no single common virulence factor has been identified in all strains.

2.6.1.1 APEC pathogenesis

E. coli is an intestinal commensal of poultry (Smith *et al.*, 2007), but some of these fecal isolates when inhaled by poultry can colonize the respiratory tract and then cause colibacillosis which can include respiratory infection as well as systemic fatal disease (Dho-Moulin *et al.*, 1999). Colonization in the trachea and the air sacs is considered the first step of systemic infection for APEC (**Figure 2.4**). Airsacculitis is a common type of infection in poultry of all ages. In addition to the respiratory route, various other infection routes have been described: neonatal infections, infections through dermal lesions, infection of the reproductive organs. Once in the blood, APEC can then disseminate to the liver and pericardium, and the infection may also lead to bacteremia and septicemia (Dho-Moulin *et al.*, 1999). A laying hen having *E. coli* induced salpingitis can have an infected egg before shell formation (Saif *et al.*, 2003). This can lead to infection and a high mortality rate among young chicks during the first few days or weeks after hatching through yolk sac infection (Saif *et al.*, 2003).

Adhesins such as fimbriae, Stg, Tsh, Yqi can contribute to APEC colonization of the respiratory tract: the trachea and the lungs (Dozois *et al.*, 2000). Carbohydrate metabolism may also contribute to the colonization of the lung or air sacs (Chouikha *et al.*, 2006). To disseminate in the bloodstream, bacteria need to translocate through the lungs and air sac interstitium. K1 capsule was shown to facilitate the translocation of APEC into the vascular system (McPeake *et al.*, 2005). When APEC disseminates in the blood, they are confronted by innate immune defenses and nutrient limitation. In this context, siderophores such as aerobactin and salmochelins, the ChuA heme uptake and Sit metal transporter sequester iron and other metals from the host environment (Caza *et al.*, 2008). In addition, protectins which help to evade the host immune system like K1 capsule, Iss, Type III secretion system, and specific O antigens can contribute to survival and virulence through resistance to the avian immune cells and the serum complement system (Mellata *et al.*, 2003).

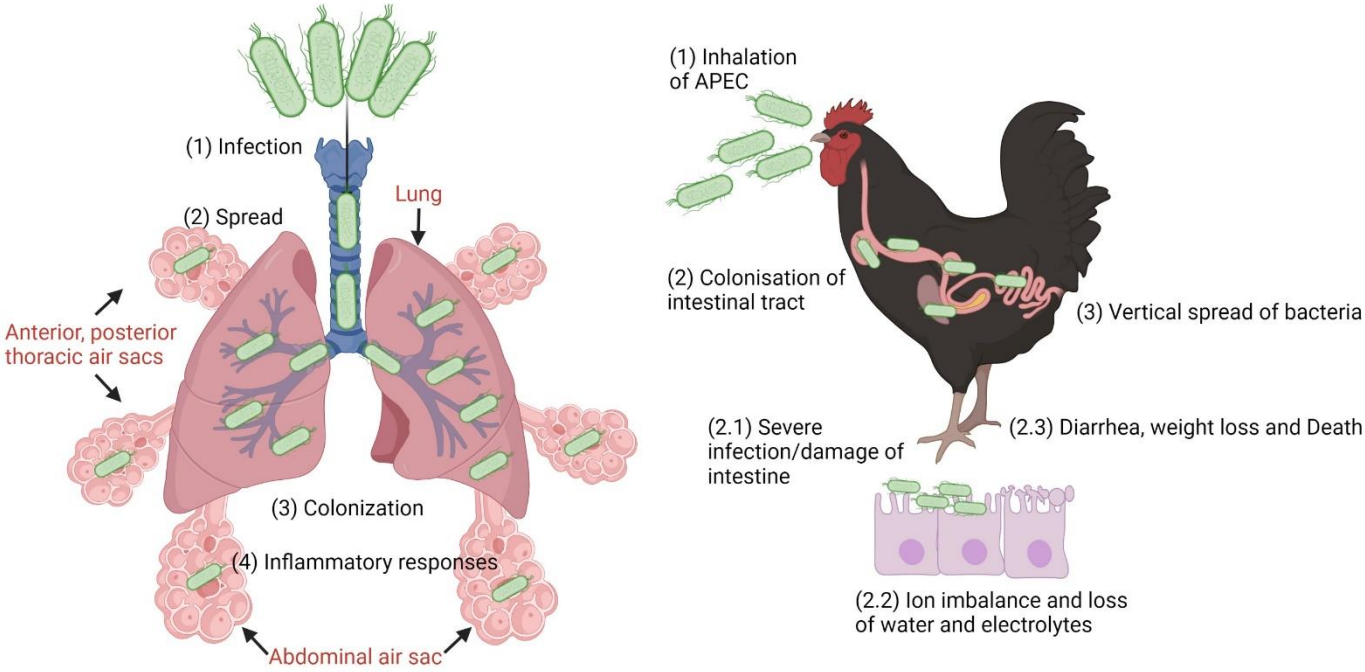

Figure 2.4 Overview of APEC infection.

APEC can reach the trachea by inhalation of contaminated aerosol particles (1). Once inside lung and airsac, the avian immune response is triggered by interactions with pathogen associated molecular patterns (PAMPS) recognized by receptors such as the Toll – like receptors (TLRs) (2). Inflammatory responses to bacterial infection will elicit macrophages and heterophils to the infected site. Inflammation will lead to tissue damage. APEC can also reach the gastrointestinal tract (2) and sometimes cause diarrhea (2.1,2.2) or invade through intestinal epithelium in presence of environmental stressors (production-related stress, immunosuppression, and concurrent infections) leading to systemic infection (2.3). APEC can be transmitted to other chickens via fecal-oral or aerosol route or vertically spread by infection of eggs (3). The figure was created with BioRender.com.

2.6.1.2 Vaccines and vaccination strategies against APEC

After registration in the EU in 2013, O78 (Poulvac® *E. coli*, Zoetis) is commercially used as a vaccine, which is a live-attenuated *aroA*-mutant of an *Escherichia coli* serogroup O78 strain (Mombarg *et al.*, 2014). The vaccine is available as a freeze-dried powder (lyophilisate) and is given as a single-dose vaccine either by spray application with chicks from one day of age or by adding to drinking water of chicks from five days of age. One important limitation of this vaccine that has been reported is a limited protection against the diversity of APEC belonging to a multitude of different serotypes (Filho *et al.*, 2013; Sadeghi *et al.*, 2018). There is therefore a need to develop a vaccine against APEC that would provide broader spectrum protection against multiple serotypes. Another

vaccination strategy is the use of autogenous killed bacterial isolates as vaccines, which can provide an important means of reducing recurring APEC infections in poultry rearing and production facilities (Koutsianos *et al.*, 2020). Autogenous vaccines have a long tradition, and the use of killed pathogenic strains as vaccines isolated from the farm of origin can provide decrease in recurring outbreaks. Both risks and benefits are associated with this technique, with the major risk being the potential transmission of viral, bacterial, and/or fungal contaminants (Lozica *et al.*, 2021). On the positive side, autogenous vaccination can provide an important protective advantage when there is a sudden outbreak, and other commercially available vaccines could not adequately protect due to heterogeneity of the APEC outbreak strains. Other techniques include the use of inactivated or killed vaccines prepared from the whole bacterial preparation combined with adjuvant and subunit vaccines prepared from outer membrane proteins, whole-cell proteins, flagellin, pilus proteins, or LPS (Chen *et al.*, 2021; Kathayat *et al.*, 2021) (**Figure 2.5**). It is important to note that although vaccination may provide some level of potential protection against colibacillosis, proper management and biosecurity in the poultry farm are paramount for maintaining good poultry health. Some of the ongoing research regarding vaccines and vaccine development against APEC are presented in **table 2.6**.

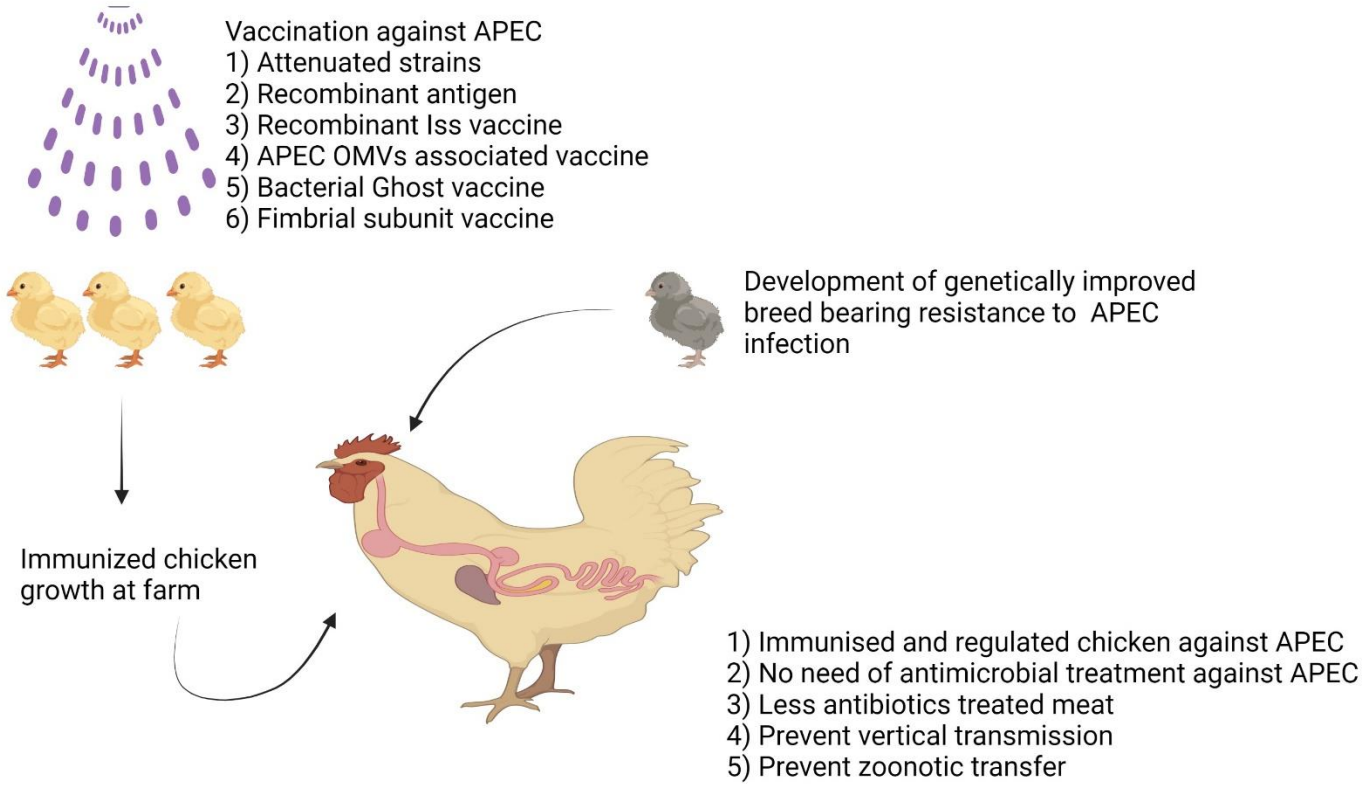

Figure 2.5 Vaccine development strategies against APEC.

Different approaches used for vaccination against E. coli in poultry include: live-attenuated vaccines (attenuation by chemical mutagenesis, or specific genetic modifications causing decreased virulence), inactivated or killed vaccines (whole bacterial cells killed by heat; chemicals like formaldehyde, acetone, alcohol, irradiation, etc.), toxoids (toxins rendered innocuous by heating or formalin), or subunit-based vaccines (prepared from immunogenic epitopes of whole-cell proteins, outer membrane proteins, flagellin, fimbriae, lipopolysaccharides), and recombinant DNA technology (DNA vaccines, mRNA vaccines). These vaccines are normally administered by spray using coarse aerosol sprayer machine or mixed in drinking water free of chlorine or other sanitizing agents to one-day old chickens or within the first week after hatching. The figure was created with BioRender.com.

Table 2.6 Avian colibacillosis treatment/vaccine strategies

Type of vaccine	Component of vaccine	Results/Observations/Outcomes	Animal model (Year)	References
Bacterial ghost of APEC+A106: D112	Design of bacterial ghost of *E. coli* O78:K80 by making porous cell wall	Aerosol vaccinated chickens challenged with APEC O78:K80 had reduced air sac lesions and less death. Vaccinated chickens showed increased levels of IFNγ, IgA and IgY.	Broiler chicken (2018)	(Ebrahimi-Nik *et al.*, 2018)
	Nucleic acid free bacterial ghost vaccine of *E. coli* O78:K80 by removing cytoplasmic content and nucleic acids.	Chickens vaccinated by injection or inhalation both showed humoral and cellular immune responses and cytokine responses. Challenge with O78:K80 showed lower lesion scores and bacterial numbers in vaccinated group.	Broiler chicken (2020)	(Soleymani *et al.*, 2020)
Liposomal inactivated APEC vaccine	Liposomal inactivated avian pathogenic *Escherichia coli*	Chickens vaccinated via eye drop or coarse spray produced anti-LPS antibodies (IgG) in serum and IgA in oral mucus.	Broiler chicken (2009)	(Yaguchi *et al.*, 2009)

	(APEC) strain containing vaccine.			
Recombinant attenuated Salmonella vaccine (RASV)	RASV producing *E. coli* common pilus (ECP) and booster dose with combination of RASV χ8025(pYA3337) and χ8025(pYA4428) or χ8025(pYA3337), RASV χ8025(pYA4428) carrying *ecp* operon genes.	Chickens vaccinated orally and challenged with APEC O2 or O78 strain via air sac. Immunized chickens after vaccination showed significantly increased levels of serum (IgY) and intestinal (IgA) antibody. Challenged chicken showed partial protection against APEC.	White Leghorns chicken (2018)	(Stromberg *et al.*, 2018)
	RASV with *Δlon, ΔcpxR,* and *ΔasdA16* and producing P-fimbriae, aerobactin receptor, and CS31A surface antigen of APEC.	Immunized chickens showed increased IgG and IgA antibodies. Chickens were challenged via air-sac route and showed partial protection against virulent APEC.	Broiler chicken (2013)	(Chaudhari *et al.*, 2013)
	RASV plus commercial probiotics supplements	White leghorn Chickens given RASV, and probiotics elicited significant serum and mucosal antibodies. When challenged with APEC virulent strains showed lower bacterial loads, and lesions of airsacculitis and pericarditis/perihepatitis.	White leghorn chicken (2020)	(Redweik *et al.*, 2020)

Outer membrane vesicle (OMV) based vaccine	Purified OMVs from O1, O2, and O78 strains to develop multi-serogroup vaccine (MOMVs).	Vaccinated chickens effectively yielded specific antibody response against each OMV antigen. They also yielded significant cellular and humoral immune responses. Immunization with MOMVs showed 100%, 90% and 100% cross-protection against challenge by O1, O2 and O78 APEC strains.	Broiler chicken (2020)	(Hu *et al.*, 2020)

2.6.2 Porcine Colibacillosis

Porcine colibacillosis are enteric infectious diseases caused by pathogenic *E. coli* which account for one of the major diseases affecting the swine industry. Colibacillosis in swine includes mainly neonatal diarrhea and post-weaning diarrhea resulting in illness and death occurring worldwide in neonatal and recently weaned pigs. Neonatal swine diarrhea is observed in piglets aged 1-4 days after farrowing, pre-weaning diarrhea within 1-2 weeks post farrowing to weaning, post-weaning diarrhea occurs 2-3 weeks after weaning with a peak rise of diarrhea from 6-8 weeks and even at 12 weeks after weaning (Fairbrother *et al.*, 2019; Francis, 1999; Kongsted *et al.*, 2018). In addition, Edema disease is usually observed after weaning and other *E. coli* infections such as mastitis, UTIs (cystitis) and septicemia are also observed. Although EHEC, EAEC, EIEC are also known to cause pathogenesis in pigs; ETEC and EPEC are major pathovars that cause serious health problems and even death in piglets around the world. Enterotoxigenic *E. coli* (ETEC) is the most important pathotype and is commonly associated with post-weaning diarrhea (PWD) and neonatal diarrhea. Cases of PWD has also been associated with EPEC (Luppi *et al.*, 2016; Nakazawa *et al.*, 1987; Vidal *et al.*, 2020). Neonatal diarrhea and PWD are the most prevalent porcine diseases, accounting for substantial economic losses worldwide (Schulz *et al.*, 2015). Common serotypes of ETEC causing neonatal and post-weaning diarrhea include O8: K88, O149: K88 and O157: K88 (Fairbrother *et al.*, 2005; Luppi *et al.*, 2016; Vidal *et al.*, 2020). In addition, many other serotypes are associated with ETEC infections in piglets. Apart from ETEC and EPEC,

Verotoxigenic *E. coli* is also known be associated with PWD, edema disease and other related infections in swine. Among verotoxigenic *E. coli* isolates of weaned pigs with enteric disease, common serotypes include O157: K17, O149:K91 and O groups 138, 139 and 141 (Gannon *et al.*, 1988; Ho *et al.*, 2013; Noamani *et al.*, 2003; Wu *et al.*, 2007).

Adhesins and enterotoxins are major virulence factors of *E. coli* causing diarrheal disease in pigs. Adhesins contribute to colonization, proliferation and development of infection and enterotoxins also play a role in colonization and development of disease (Luppi *et al.*, 2016). ETEC strains can produce several different fimbrial adhesins. Fimbriae in ETEC are divided into four main types: F4 (K88), F5 (K99), F6 (987P), and F41. The ETEC strains also produce LT and ST (STa and STb) enterotoxins. It has been reported that ETEC strains producing F4 fimbrial adhesins and producing LT and ST enterotoxins are responsible for causing diarrhea in neonatal, pre- and post-weaning pigs (Dubreuil *et al.*, 2016; Hartadi *et al.*, 2020). In addition, EPEC strains with F41 and F18 fimbriae and producing STb enterotoxin STb are known to cause swine diarrhea. Moreover, VTEC strains with F4 (K91) fimbriae and LT and ST enterotoxins are also reported to cause infections in pig (Gannon *et al.*, 1988; Kim *et al.*, 2010b; Osek, 2000).

2.6.2.1 Resistance to multiple antibiotics in *E. coli* causing diarrhea in swine.

Swine production is one of the major sources of animal meat production. Over the years the presence of bacterial diseases has resulted in increased use of antibiotics to prevent such infections and reduce economic losses and improve animal health. Wide use of antibiotics has consequences, and there is a global concern in increasing antibiotic resistance. It has been reported that there is a high level of antibiotic resistance in porcine *E. coli* isolates from China (Abdalla *et al.*, 2021; Liu *et al.*, 2022; Peng *et al.*, 2022; Schoenmakers, 2020; Wang *et al.*, 2020; Yang *et al.*, 2019). Antibiotic resistance in *E. coli* is also prevalent on pork farms across Europe, including Spain (Abreu *et al.*, 2019; Vidal *et al.*, 2020), and other pork exporting countries like Denmark, France, Italy, and Sweden are also found to be multi-drug resistant to antibiotics such as ampicillin, streptomycin, sulphonamide, tetracycline, and trimethoprim (Österberg *et al.*, 2016).

2.6.2.2 Need of vaccines and vaccine strategies against Porcine pathogenic *E. coli.*

The pork industry is one of the most important meat industries in terms of numbers and biomass. The UN Food and Agriculture Organization has predicted that the pork meat industry will

experience a strong growth among all meat industries with an expected increase of 8.6% by 2030 and 12.7% by 2050. It is therefore crucial to understand and structure sustainable growth of the pork production industry in terms of animal health, antibiotic resistance, and alternative routes to antibiotic through the development and implementation of vaccines and other approaches to improve porcine health and provide a high-quality consumer product globally.

Since adhesins and enterotoxins are vital for ETEC pathogenesis and virulence, various approaches have been developed to inhibit initial stages of ETEC pathogenesis by developing anti-adhesin and anti-toxin-based vaccines (Melkebeek *et al.*, 2013; Nadeau *et al.*, 2017). Over the past decade, multiple vaccination strategies have been developed. For example, a live-attenuated *S. typhimurium* Δ*lon* Δ*cpxR* Δ*asd* strain has been used to deliver ETEC fimbriae in order to promote an anti-adhesin based vaccine immune response in swine. This vaccine showed no clinical signs of diarrhea in the vaccinated and challenged group and generated increased levels of IgG and IgA in both serum and colostrum (Hur *et al.*, 2012). Some porcine ETEC strains produce enterotoxins, but use fewer common types of fimbriae, so it is important to design a vaccine that can provide a large spectrum of protection against multiple types of porcine pathogenic *E. coli*. One such example is a live-attenuated *E. coli* strain expressing an adhesin-toxoid fusion antigen that has been shown to elicit a good systemic and mucosal antibody response and protect piglets from ETEC associated diarrhea (Ruan *et al.*, 2013). Another group designed a multivalent vaccine candidate comprising STa-LTB-STb (SLS) toxin-based antigens, and two fimbrial proteins, which showed significant protection against an ETEC strain (Zhang *et al.*, 2018). Likewise, a peptide-based vaccine constructed by genetically fusing nucleotides encoding peptides for fimbria and toxins to obtain a tripartite adhesion-adhesin-toxoid chimeric antigen, were given to piglets and they provided protected against a challenge using a fimbrial/LT/STb producing ETEC strain (Ruan *et al.*, 2011). This study demonstrated that multiple adhesin antigens and multiple toxin antigens could be expressed simultaneously within a single recombinant fusion protein. In the future, non-pathogenic *E. coli* isolates from farm that expresses a tripartite antigen could also be used to develop live attenuated vaccine against porcine ETEC. Along with these, other strategies such as an oral fimbrial subunit vaccine, live oral vaccine, encapsulated subunit, and other live vaccine candidates are described in detail in the following reviews (Dubreuil, 2021; Fairbrother *et al.*, 2019; Fairbrother *et al.*, 2005; Melkebeek *et al.*, 2013).

Another interesting strategy that is being considered is a parenteral vaccine which is based on the presence of lactogenic maternal antibodies via parenteral immunization. The entero-mammary route allows the oral maternal immunization to elicit a mucosal and systemic immune response to reach the gut of the suckling piglet with humoral and cellular components with effector

activity (Dubreuil, 2021). This will provide protection against infection by either maternal antibodies and leukocytes present in the suckling infants or by acquired specific piglet immunity through the sow. Research has suggested that suckling piglets born from immunized sows with Sta toxoid fusion antigens were protected from a challenge with a STa-positive ETEC strain (Zhang *et al.*, 2010). Another vaccine candidate containing a Sta toxoid fusion and chemical conjugates was used to immunize sows, and the suckling piglets passively acquired anti-STa IgG and IgA antibodies and were better protected from challenge with a STa positive ETEC strain (Matías *et al.*, 2017; Seo *et al.*, 2019). Some popular commercially available vaccines are manufactured by Elanco and Vencofarma and are comprised of inactivated ETEC bacterins which are administered to pregnant pigs two weeks before farrowing.

Overall, some approaches to vaccination against porcine *E. coli* have shown promise against some common types of strains causing swine diarrhea. However, due to the diversity of pathogenic *E. coli* certain strains are still a problem to the industry. Importantly, a global and integrative approach is needed to regulate and monitor multiple aspects from sanitation, feed regimen, antibiotic usage, and vaccine strategies to maintain and improve the production of sustainable pork production industry worldwide.

2.6.3 Bovine Colibacillosis

Bovine colibacillosis is an infection caused by ETEC strains. Although EPEC, EHEC and VTEC strains are commonly present in the bovine intestinal tract (Arimizu *et al.*, 2019), they are generally considered non-pathogenic to cattle (Durso *et al.*, 2004). Enteric colibacillosis is usually observed in young calves that are from 2-10 days old (Haggard, 1985). Stress can also lead to ETEC-associated diarrhea in older calves. During the infection, calves suffer from profuse and watery diarrhea, become dehydrated, depressed, anorexic, do not suckle and may die rapidly. Depending on the severity of symptoms calves frequently die in 3 to 5 days (Bashahun *et al.*, 2017). Colibacillosis outbreaks in cattle have been associated with multi-resistant strains under poor sanitation conditions and with susceptible calves in the herd population (Rigobelo *et al.*, 2006; Shuguang *et al.*, 2017). It has been shown that calves of first-calf heifers are more susceptible to infection mainly due to presence of lower levels of immunoglobulin in colostrum and production of less colostrum itself (Acres, 1985; Bush *et al.*, 1980). Calves are also known to develop septicemic colibacillosis due to invasive serotypes of ETEC that can enter the blood stream, cause a systemic infection and rapid death due to septicemia (Besser *et al.*, 1985; Ercan *et al.*, 2016). Generally, ETEC strains adhere to the ileum of calves and release toxins to induce severe diarrhea. Septicemic

E. coli strains can cause systemic infection and, in some cases, EHEC and VTEC strains produce shiga-toxin that destroy gut microvilli and can cause hemorrhagic diarrhea in 15 to 30 day-old calves (Bazeley, 2003; Moxley *et al.*, 2010).

Bovine ETEC isolates from diseased calves commonly produces K99 (F5) adhesin and heat stable (STa or STb) or heat liable (LT1 or LT2) enterotoxins. It has been reported that out 173 isolates of ETEC 49% of strains produced toxins where 53 isolates harbored Sta toxin and 9 contained both STa and LT toxin encoding sequences. Interestingly, 9 isolates harbored shiga-toxin genes (Baldo *et al.*, 2020; Choi *et al.*, 2001; Dubreuil, 2008; Picco *et al.*, 2015; Rigobelo *et al.*, 2006; Salvadori *et al.*, 2003). There is also a high incidence of multiple drug resistance in bovine ETEC isolates throughout the world (Algammal *et al.*, 2020; Ali *et al.*, 2021a; Shuguang *et al.*, 2017; Srivani *et al.*, 2017; Tadesse, 2020) (Fesseha *et al.*, 2022) (de Verdier *et al.*, 2012; Hariharan *et al.*, 2004; Srivani *et al.*, 2017). With an increased concern due to multiple antibiotic resistance, there is a greater interest in vaccines and other preventative measures to reduce incidence of colibacillosis in calves (See **table 2.7** which lists such vaccine strategies).

2.6.4 Mastitis in cattle and swine

Mastitis is a disease characterized by intramammary infections caused mainly by microorganisms and in some cases worsened by physical trauma that results in persistent inflammation of udder and mammary tissue (Bradley, 2002). Microorganisms such as viruses, fungi, mycoplasma, and numerous bacterial species can cause mastitis. *E. coli* are a common cause of mastitis (Gomes *et al.*, 2016; Khan *et al.*, 2006; Zhao *et al.*, 2008). Mastitis in swine is one of the causes for maternal and pre-weaning piglet mortality (Gerjets *et al.*, 2009). *E. coli* is known to be one of the leading causes of inflammatory infection of mammary glands in sows, resulting in abnormal and decreased milk production and even death in severe cases (Goulart *et al.*, 2022). Among different pathotypes of *E. coli*, a novel MPEC (Mammary pathogenic *E. coli*) is one of the most common causative agents of coliform mastitis (Goulart *et al.*, 2022). MPEC strains have been evolved by acquiring virulence factors that promote colonization and invasion of the mammary glands and increase survival in milk (Blum *et al.*, 2018; Blum *et al.*, 2015; Shpigel *et al.*, 2008).

Due to excessive use of antibiotics to treat bovine mastitis, there has been increased concern of the presence of antibiotic resistant bacteria on dairy farms and in milk and dairy products (Ali *et al.*, 2017; Saini *et al.*, 2012; Tahar *et al.*, 2020; Todorović *et al.*, 2018). Therefore, to overcome this challenge different vaccination approaches to prevent coliform mastitis have also been tested. Commercially, mutant strains of *E. coli* O111:B4 (J5) (Hogan *et al.*, 1992) and *Salmonella*

Typhimurium Re-17 (McClure *et al.*, 1994) are being used to immunize cows against coliform mastitis. In addition, recombinant OmpA (outer membrane protein A) is also being studied as a potential vaccine candidate to prevent infection against mastitis-associated *E. coli* (Gomes *et al.*, 2016; Liu *et al.*, 2021). Other vaccination strategies against mastitis caused by organisms other than *E. coli* are also described in **table 2.7**.

Table 2.7 Reports concerning bovine colibacillosis treatment/vaccine strategies.

Type of vaccine	Component of vaccine	Results/Observations/Outcomes/Year	References
ScourGuard® 4K	Cocktail of inactivated bovine rotavirus, coronavirus, and *E. coli* bacterin	Vaccination of healthy pregnant cows prevented diarrheal disease in calves bovine ETEC bearing K99 pili, bovine rotavirus (serotypes G6 and G10), and coronavirus (2021)	(Ali *et al.*, 2021b) (Zoetis, USA)
ScourGuard® 4Kc	Cocktail of inactivated bovine rotavirus, coronavirus, *Clostridium perfringens* type C and *E. coli* bacterin-toxoid	Vaccination of healthy pregnant cows prevented diarrhea caused by bovine rotavirus (serotypes G6 and G10), coronavirus, ETEC and *C. perfringens* in calves given colostrum from vaccinated mother (2021)	(Ali *et al.*, 2021b) (Zoetis, USA)
Bolus and Dual-force gel	Contains passive antibodies against diarrheal pathogens	Single dose administered after birth protects calves from *E. coli* and coronavirus infection (2021)	First Defense
Tri-Shield First defense	Contains passive antibodies against diarrheal pathogens. Should administered with maternal colostrum	Single dose administered after birth provides passive immunity against K99+ *E. coli*, coronavirus, and rotavirus (2021)	First Defense
First defense Technology	Hyper-immunized colostrum antibodies	Antibodies that neutralize *E. coli* and coronavirus and provide instant immunity (2021)	First Defense
Bioniche® vaccine	A Type III secretion system-based vaccine	Reduces *E. coli* O157:H7 (EHEC) growth and colonization in cattle (2013)	(Walle *et al.*, 2013) Bioniche

Epitopix® vaccine	Siderophore receptor and porin protein (SRP) vaccine	Reduces *E. coli* O157:H7 (EHEC) growth and colonization in cattle (2018)	(Fingerman *et al.*, 2018)
Fencovis® vaccine	Administered to pregnant cows to provides passive immunity to newborn calves via maternal colostrum	Active immunization of cows stimulates development of antibodies against *E. coli* F5, rotavirus and coronavirus and prevents neonatal diarrhea (2022)	(Ingelheim, 2022a)
J-VAC® vaccine	Broad spectrum adjuvanted bacterin-toxoid	Prevents bovine mastitis caused by *E. coli* and endotoxemia caused by *E. coli* and *Salmonella Typhimurium* (2022)	(Ingelheim, 2022c)
Bar-Guard-99™ vaccine	Utilizes whole cell antibodies	Provide rapid passive immunity against *E. coli* K99 and diarrheal diseases (2022)	(Ingelheim, 2022b)
ENVIRACOR® J-5 vaccine	Bacterin-based vaccine	Controls clinical signs related to bovine mastitis caused by *E. coli* (2021)	Zoetis, USA
BOVILIS® J-5 vaccine	Endotoxin-based vaccine	Prevents milk loss, culling and death related to bovine mastitis caused by *E. coli* (2021)	MERK animal health
DNA and subunit-based vaccine	Lipopolysaccharide based pcwaaF (DNA vaccine) and rwaaF (recombinant waaF subunit vaccine)	Greater IgG, IL-2, IL-4, and IFN-γ, and fecal sIgA. Mice survival better post-challenge with mastitis causing *E. coli* (2022)	(Wang *et al.*, 2022a)
Proteo-liposome based vaccine	Proteo-liposome extracted from bovine mastitis clinical isolate (RM5870)	Significant level of IgG, IgG1 and IgG2a, IgA. Improved survival rates of mice post-challenge with *E. coli* causing mastitis. Reduced bacterial loads, inflammation, and tissue damage in mammary glands (2022)	(Quiroga *et al.*, 2022)

2.7 Conclusions of review article

The diversity of pathogenic *E. coli* which collectively includes distinct pathotypes of importance for both human and animal health has been and remains an important challenge for the development

of effective vaccines. Identifying protective and conserved antigens to prevent specific types of *E. coli* disease is complicated by the different types of diseases as well as the heterogeneity of gene content contributing to the pathogenic potential among, and even within, pathotypes associated with distinct disease syndromes in either humans or other animals. Thus far, for vaccine candidates to prevent human infections, almost all the studies have been in animals and the few human studies conducted to date have either been failures, generated inconclusive data, or have not been further pursued. Based on the aforementioned diverse nature of *E. coli* virulence factors, one can predict that an “ideal” vaccine that can prevent multiple types of *Escherichia coli* infection and disease is unlikely. However, development of different vaccine types and innovative immunization strategies appears promising. A major hurdle in overcoming *E. coli* pathotypes in some cases is the presence of multiple serotypes that are responsible for either human or animal infections. Surface-exposed components are often attractive and effective candidates or vaccine development, but in the case of *E. coli* vaccines, particularly in humans, selection of targets more specific to pathogenic strains may be a more favorable approach, than targeting highly conserved antigens, considering that *E. coli* strains are also common members of the intestinal microbiota. As such, generation of an immune response against highly conserved antigens in most *E. coli* and other enterobacteria, may be protective against some types of infection, but may also affect the host intestinal mucosal immune response and colonization of commensal *E. coli* in the intestinal tract. Such aspects of effects of selection of highly conserved vs. more patho-specific antigens need to be more fully investigated in both animal models and humans in future studies. In addition to the vaccine development strategies presented herein, it will be of interest as well to investigate how other novel vaccine approaches such as mRNA vaccination may provide protection against certain pathotypes of *E. coli*. However, use of such vaccines may be limited due to cost or issues with vaccine stability and the requirement of a cold chain particularly for vaccination of populations in outlying regions and in developing countries where endemic infections such as diarrhea caused by intestinal pathogenic *E. coli* and other pathogens remain a global health challenge. Apart from vaccination campaign, different preventive strategies such as improved sanitation and hygiene, access to safe drinking water, exclusive breastfeeding, optimal nutrition, and vaccines against other pathogens (rotavirus and measles) are equally important. In any case, continued development of new strategies for *E. coli* vaccine discovery is warranted in the context of its antibiotic resistance. Hopefully such strategies will lead to the development of more effective means to reduce the incidence of the spectrum of infectious diseases that *E. coli* can cause in both humans and animals of agricultural importance which remain a globally important economic and public health burden.

2.8 Acquisition of virulent factors in *E. coli.*

Acquisition of genes contributing to virulence and genomic plasticity of *E. coli* is one of the major reasons underlying its diverse pathogenic spectrum. The major mechanisms for the acquisition of virulence factors are horizontal gene transfer and spontaneous mutation. Horizontal gene transfer accounts for the gain of new genes encoding for virulence factors. In addition, transfer of metabolic genes to adapt to novel energy sources have been reported (Burmeister, 2015; Pál *et al.*, 2005). Spontaneous mutation accounts for the modification or loss of existing genes. Such adaptations are crucial for bacteria to develop resistance to lethal factors such as bacteriocins and antibiotics. Altogether, the genomic plasticity of some bacteria such as *E. coli* has promoted it's adaptability to survive, colonize and reproduce in novel ecological niches, varying environmental conditions, and compete with other microorganisms (Bielaszewska *et al.*, 2007; Dobrindt *et al.*, 2001).

Horizontal gene transfer is defined as the transfer of genes from different species of distinct strains to another. These processes are mediated by mobile genetic elements such as transposons/insertion sequences (IS), bacteriophages and plasmids. Genes encoding virulence factors, metabolic genes and antibiotic resistance genes present in these elements can be horizontally transferred between species/strains (Frost *et al.*, 2005; Partridge *et al.*, 2018; Rankin *et al.*, 2011). Using transposition mechanism, the transposons and IS can move and multiply independently. IS and parts of DNA between these elements can be translocated into other strains via replicon insertion or fusion (Arnold *et al.*, 2022; Bichsel *et al.*, 2013). Bacteriophages are viruses of bacteria that in some cases incorporate their genome into bacteria and utilize the host machinery to replicate and reproduce. During this process bacteriophages may introduce bacterial DNA by transduction (Penadés *et al.*, 2015; Schneider, 2021). Plasmids are extra-chromosomal replicating DNA reside within bacterial cells. Plasmids carrying various virulence and metabolic genes can be transferred between bacteria via conjugation (Harrison *et al.*, 2012; Johnson *et al.*, 2009; Reid *et al.*, 2022; Skyberg *et al.*, 2006). Depending on the circumstances and advantages acquisition of plasmid encoded genes may provide, bacteria may reject or retain the plasmids to provide certain fitness advantages. It has been reported that pathogenicity islands (PAIs) which are usually absent in commensal *E. coli* were likely transferred horizontally via the aforementioned mechanisms to result in the emergence of different pathotypes of *E. coli* (Dozois *et al.*, 1999).

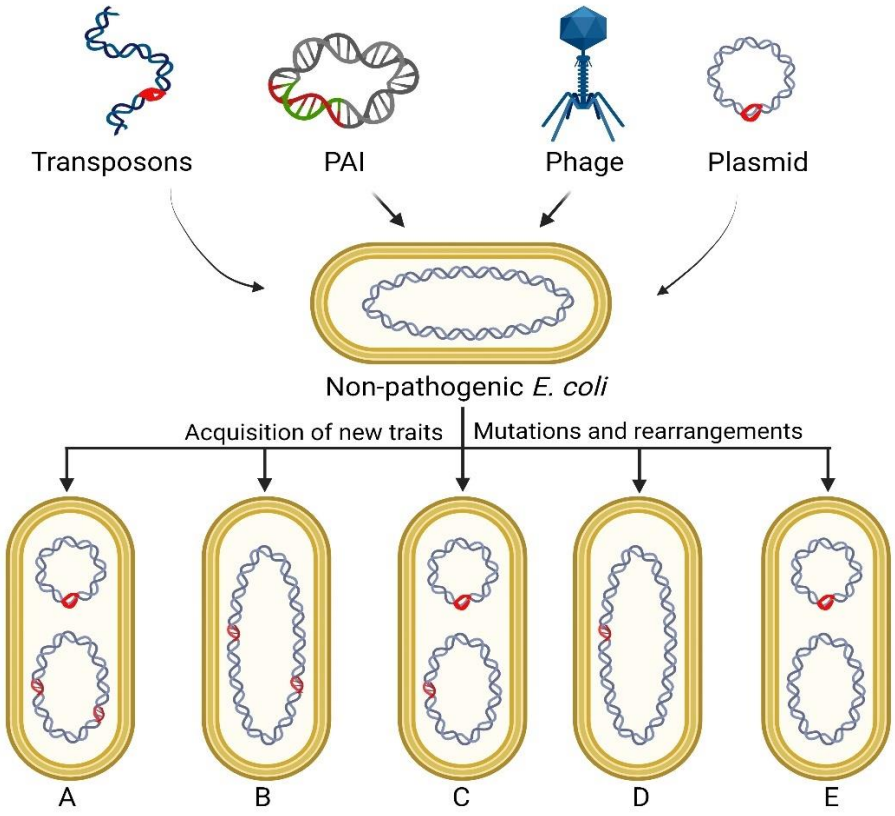

Figure 2.6 Acquisition of virulent factors by horizontal gene transfer.

Commensal *E. coli* and pathogenic *E. coli* in different host can acquire new virulence traits with the help of mobile elements such as transposons and pathogenic island transfer where bacteria can transform naked DNA to non-pathogenic bacteria. Phage leads to transduction and carry genome from one bacterium to another during its propagation. Virulence plasmids can be conjugated and transferred from one bacterium to another to acquire virulence factors. A) Gain of virulence plasmid and genes in the pathogenicity islands (multiple locations) of chromosome, B) Attainment of virulence genes at multiple locations of genome, C) Acquisition of virulence genes at single location and by plasmid, D) Gain of virulence gene at single location of bacterial genome, and E) Uptake of virulence plasmid (Red color reflects the virulence gene) (Adapted/modified from (Kaper *et al.*, 2004)). The figure was created in Biorender.com.

Spontaneous mutations such as insertions, deletion and point mutations can lead to the change in function of genes either by providing altered or improved activity, changes in expression levels, or inactivation of genes. Beneficial spontaneous mutations are have also occur in *E. coli* which have permitted it to adapt to improved colonization of host tissues or fitness advantages during infection of different host species (Gebisa *et al.*, 2019; Sokurenko *et al.*, 1999). (**Figure 2.6**)

2.9 Some virulence traits of extraintestinal pathogenic *E. coli* from different animal and human host are shared.

Although there is substantial genetic diversity among the various pathotypes of ExPEC, some of them share similar virulence factors. Commonly identified virulence traits of ExPEC can include:

iron/metal acquisition system, adhesins, toxins, invasins, and components that promote resistance to serum and survival against phagocytes (Sarowska *et al.*, 2019). Iron/metal acquisition systems are one of the critical traits for growth of ExPEC in extraintestinal sites. Small iron-sequestering molecules called siderophores are important for ExPEC persistence and growth during infection. The main siderophore systems in ExPEC pathotypes include: salmochelins (often present in APEC, UPEC and MNEC); aerobactin (a siderophore present in APEC and UPEC); the yersiniabactin siderophore (present in UPEC and MNEC), and the enterobactin siderophore is common to most *E. coli* including commensal isolates. In addition to siderophores SitABC is a metal transport system, present in APEC and UPEC; and ChuA and Hma are heme acquisition systems (present in MNEC and UPEC) (Martinez *et al.*, 1990; Mellata *et al.*, 2003; Michaelis *et al.*, 1986; Nataro *et al.*, 1987; Payne, 1993) (**Figure 2.7**).

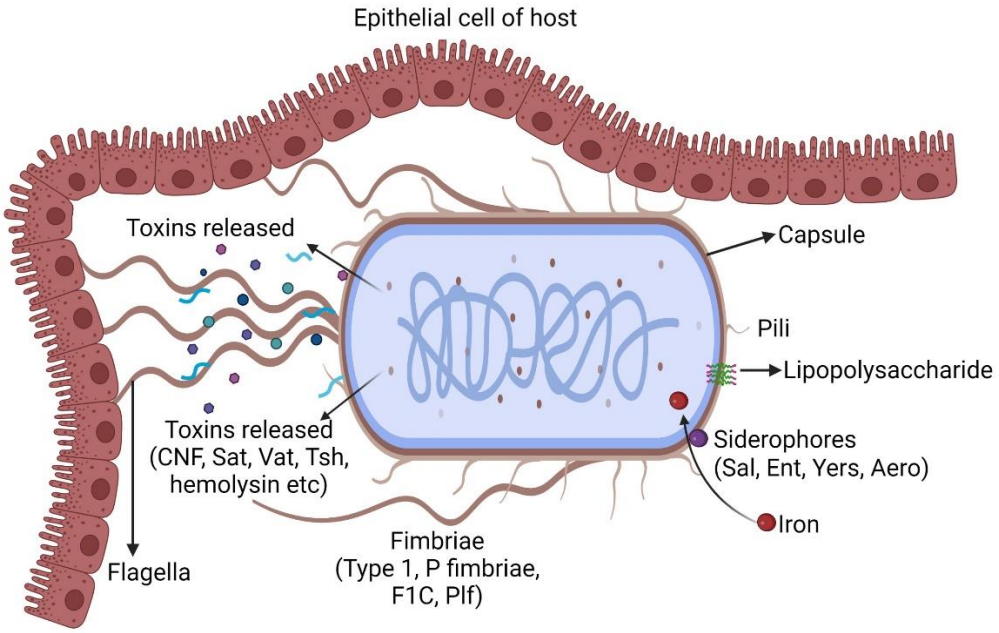

Figure 2.7 Shared Virulence traits of ExPEC

(CNF: Cytotoxic necrotizing factor, Sal: Salmochelins, Yers: Yersiniabactin, Aero: Aerobactin, Sat: Secreted autotransporter toxin, Vat: Vacuolating autotransporter toxin, Tsh: temperature-sensitive hemagglutinin, Plf: P-like fimbriae). The figure was created in Biorender.com.

Adhesins are essential during the initial attachment of bacteria to host mucosal and epithelial surfaces and establishment of infection. They can also contribute to persistent colonization and systemic infection (Sarowska *et al.*, 2019). In ExPEC , adhesins that contribute to virulence include: type 1 fimbriae, that can contribute to virulence of some APEC, UPEC and MNEC (Klemm *et al.*, 2010); P fimbriae, present in in some APEC, UPEC and MNEC (Klemm *et al.*, 2010; Lane *et al.*,

2007); Dr fimbriae that recognize receptors in kidney during systemic infection of UPEC (Goluszko *et al.*, 1997); S fimbriae that promote attachment of UPEC strains to kidney and epithelium; F1C fimbriae that were shown to mediate UPEC adherence to renal epithelial cells; Curli, an adhesin that was shown to mediate colonization of chicken trachea in APEC; the antigen 43 autotransporter and Iha outer membrane protein that have been shown to contribute to UPEC virulence; and autotransporter Tsh present in some APECstrains (Sarowska *et al.*, 2019; Sora *et al.*, 2021).

Protectins such as increased serum survival (ISS) and capsular polysaccharides present in APEC, UPEC and MNEC strains were shown to be important for virulence as they protect bacteria killing from phagocytosis and can contribute to bacterial colonization and systemic infection (Nolan *et al.*, 2003a; Sora *et al.*, 2021). Invasins such as transfer protein (TraT) that evade complement activity and IbeA that can mediate bacterial spread hqve also been reported to contribute to virulence in both APEC and MNEC (Ananias *et al.*, 2008; Cunha *et al.*, 2014). Several outer membrane proteins that can promote resistance to complement are also present in most APEC, UPEC and MNEC strains (Nielsen *et al.*, 2020). Altogether such protectins and invasins promote bacterial persistence by diminishing the effectiveness of host immune defense mechanisms.

Toxins are important virulence factors that can damage host cells and lower immune cellular and humoral defenses. Toxins such as hemolysins encoded by *hlyA* vacuolating cytotoxin (Vat); secreted autotransporter toxins (Sat); other autotransporter toxins such as Tsh, Sha, TagBC; cytholethal destending toxins that inhibit the host cell division cycle; cytotoxic necrotizing factors that modify cytoskeletal organization are known to be present in some ExPEC strains (Ghorbani *et al.*, 2021; Habouria *et al.*, 2019; Maluta *et al.*, 2014b). Altogether, these toxins can weaken the host system enabling bacteria more effectively colonize host tissues and cause disease (Kathayat *et al.*, 2021).

Despite having similar virulence factors in some cases, certain subgroups of ExPEC have unique mechanisms of causing infection in certain host species. However, some APEC strains are highly similar to UPEC or MNEC strains associated with infections in humans (Bélanger *et al.*, 2011; Ewers *et al.*, 2007). Common virulence factors, and both phylogenetic clonality and shared serogroup antigens of some APEC are near identical to human ExPEC strain, suggesting these groups of strains may have a broad range of host infectivity and zoonotic potential. This is further supported by the fact that APEC strains were shown to be able to cause UTI in mice and neonatal meningitis in rat pups (Jakobsen *et al.*, 2010; Tivendale *et al.*, 2010). Conversely, ExPEC strains isolated from humans were also shown to be virulent for poultry in chicken infection models (Moulin-Schouleur *et al.*, 2007). Therefore, overlapping of virulence traits support the potential for cross-

species transmission of APEC from food sources, specifically poultry, as a source of ExPEC for human diseases like UTIs and neonatal meningitis.

Therefore, overlap of virulent ability of these strains is posing a big challenge and potential to study the comprehensive epidemiological crosslink concerning the transmission of APEC from food source specifically poultry as a source of ExPEC for human diseases like UTIs and neonatal meningitis.

2.10 Serine protease autotransporters of *Enterobacteriaceae* (SPATEs) from the avian pathogenic *E. coli* (APEC).

Autotransporter (AT) proteins are virulent factors secreted by pathogenic *Escherichia coli*. The AT family comprises a large group of secreted polypeptides with more than 1000 identified proteins (Henderson *et al.*, 2004). Depending on their unique domain architecture, ATs can be categorized into three main groups: self-associating autotransporters (SAATs), Serine Protease Autotransporters of *Enterobacteriaceae* (SPATEs) and trimeric AT proteins (TAAs) (Habouria *et al.*, 2019; Wells *et al.*, 2010). SAATs have distinct characteristics of being glycosylated proteins. They include proteins such as AIDA-I, TibA, and Ag43 (Côté *et al.*, 2013). TAAs can mediate adherence to host cells and extracellular matrix proteins through a passenger domain that is exposed at the surface of the cells. TAAs include proteins such as UpaG and EhaG (Totsika *et al.*, 2012; Valle *et al.*, 2008). SPATEs are ATs that demonstrate a catalytic serine protease domain. Across different pathotypes of *E. coli* SPATEs include Vat, TagBC, Sha, Sat, Tsh, EspC, SepA, SigA, and TleA (Pokharel *et al.*, 2019). With respect to their functions, SPATEs have been divided into 2 subtypes: class I and class II SPATEs. Class I SPATEs represent cytotoxic proteins and class II SPATEs comprise mainly immunomodulatory proteins (Ruiz-Perez *et al.*, 2014). The SPATE proteins Vat (vacuolating autotransporter protein); Tsh (temperature sensitive hemagglutinin); Sha (serine-protease hemagglutinin autotransporter); TagB and TagC (protein encoded from tandem autotransporter genes *B* and *C*) (Habouria *et al.*, 2019); and Pic (protein involved in colonization) are reported to be expressed as virulence factors in APEC and human ExPEC (UPEC and NMEC). Distribution of SPATEs among various pathogenic *E. coli* are shown in **figure 2.10**.

2.10.1 AT secretion system

Bacterial secretion of AT proteins involves the type V secretion system (T5SS) pathway (AT secretion pathway). T5SS through which AT are secreted are divided into 5 subgroups: Va, Vb, Vc, Vd and Ve; i) Va is the secretion system for the monomeric ATs such as SPATEs , ii) Vb is for the two-partner secretion system, iii) Vc is the secretion system for the trimeric ATs, iv) Vd secretion

system is for the autotransporter proteins homologous to Va and Vb, v) Ve is the secretion systems for the invasins and intimins (Leo *et al.*, 2012; Ruiz-Perez *et al.*, 2014; Salacha *et al.*, 2010a).

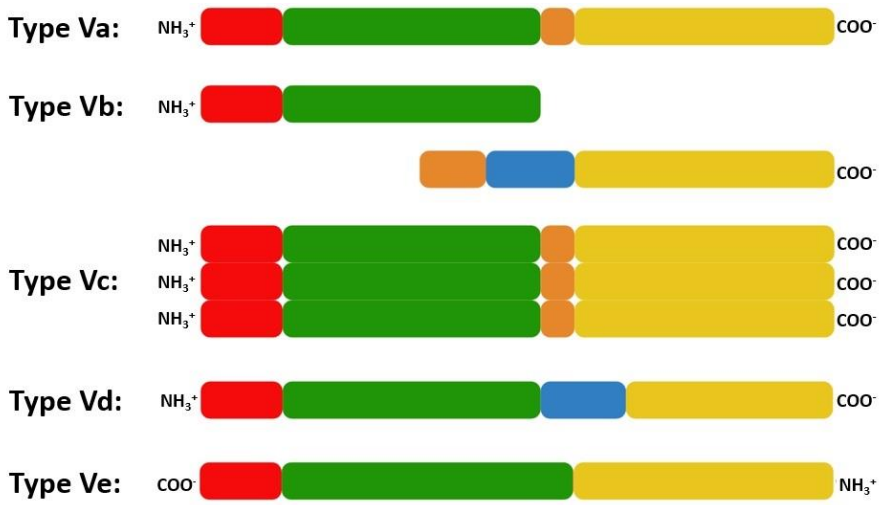

Figure 2.8 Schematics representing different subclasses of T5SS for autotransporter proteins.

The label shows the N-terminal region and C-terminal region and the orientation for each subtype. The domains were represented as colored block corresponding to: Red (signal peptide), green (passenger domain), blue (PORTA domain), orange (linker domain), yellow (translocation domain). (Adapted/modified from (Pokharel *et al.*, 2019)). The figure was created in Biorender.com.

The illustration above (**Figure 2.8**) depicts the difference in the distribution of domains of each of the subtypes of AT proteins. Type Va, possess the N-terminal signal peptide and passenger domain connected to a C-terminal translocation domain by a linker domain (Henderson *et al.*, 2004). Vb ATs are split variants of Va system, and they were described as a two-partner secretion system, where different polypeptide chains represent the passenger domain and translocation domains. In addition, the translocation domain also contains periplasmic polypeptide transport associated (POTRA) motifs (Jacob-Dubuisson *et al.*, 2001). In contrast to all other ATs, type Vc possess polypeptide chains in trimeric form, hence they are described as trimeric autotransporter adhesins (Linke *et al.*, 2006). Type Vd are similar to Va but have varied linker domains between the C-terminal translocation domain and N-terminal signal peptide and passenger domain where Vd contains an additional periplasmic domain that is similar to domains of subtype Vb (Salacha *et al.*, 2010b). Distinctively, Ve ATs have domain organization is inverted wherein the translocation domain is at the N-terminal and the signal and passenger domains are at the C-terminal (Leo *et al.*, 2012).

2.10.2 Sec-dependent export of autotransporter proteins via inner membrane

Autotransporter proteins use the sec-dependent pathway for export into the periplasmic space. Export of ATs can occur either co-translationally or via AT synthesis into the cytoplasm (Henderson *et al.*, 2004; Sijbrandi *et al.*, 2003). During export, the signal peptide is cleaved, and the passenger and translocation domains are directed towards a β-barrel assembly machinery (Bam) complex using chaperones proteins such as DegP, SurA and Skp. The Bam complex then catalyzes the insertion and formation of β-barrel from the translocation domain. The passenger domain is then released into the external environment via the β-barrel. A "hybrid barrel model" has been proposed for this pathway, where passenger domain secretion is independent of ATP and folding of the C-terminal of the passenger domain provides energy for translocation (Pokharel *et al.*, 2019).

2.10.2.1 Step by step mechanism of ATs secretion

i) All the ATs contain three major domains: N-terminal signal peptide, passenger domain, and β-domain (**Figure 2.8**). The signal peptide directs movement of AT proteins from cytoplasm to periplasm via the inner membrane Sec translocator. This signal peptide has a tripartite organization of N-terminal (n), hydrophobic (h) and cleavage (c) sites where the 'h' region is crucial for targeting and insertion of ATs into the membrane (Martoglio *et al.*, 1998; Michaelis *et al.*, 1986). In *E. coli,* Sec mediated export of proteins involves two unique pathways. One pathway is the SecB/SecA pathway where SecB, a chaperone, preserves and transfers the protein to SecA in a 'translocation-competent state' that prevents the premature aggregation or improper folding of protein (Randall *et al.*, 2002). Another pathway is the signal recognition particle (SRP) pathway where the SRP nucleoprotein complex interacts with the 'h' region of the signal sequence and mediates co-translational targeting from the ribosome to the translocon (Valent *et al.*, 1998).

ii) When the AT proteins (with passenger and β-domain) are secreted into the periplasm, they are susceptible to the degradation by periplasmic proteases, immature folding, and aggregation. Various periplasmic chaperones such as SurA, DegP, PpiD and Skp interact to prevent misfolding (Behrens *et al.*, 2001; Chen *et al.*, 1996; Dartigalongue *et al.*, 1998). In addition, the localization of proteins in the periplasm is ephemeral and their translocation to the outer membrane happens consecutively once proteins are processed through the inner membrane (Ruiz-Perez *et al.*, 2009) (**Figure 2.9**).

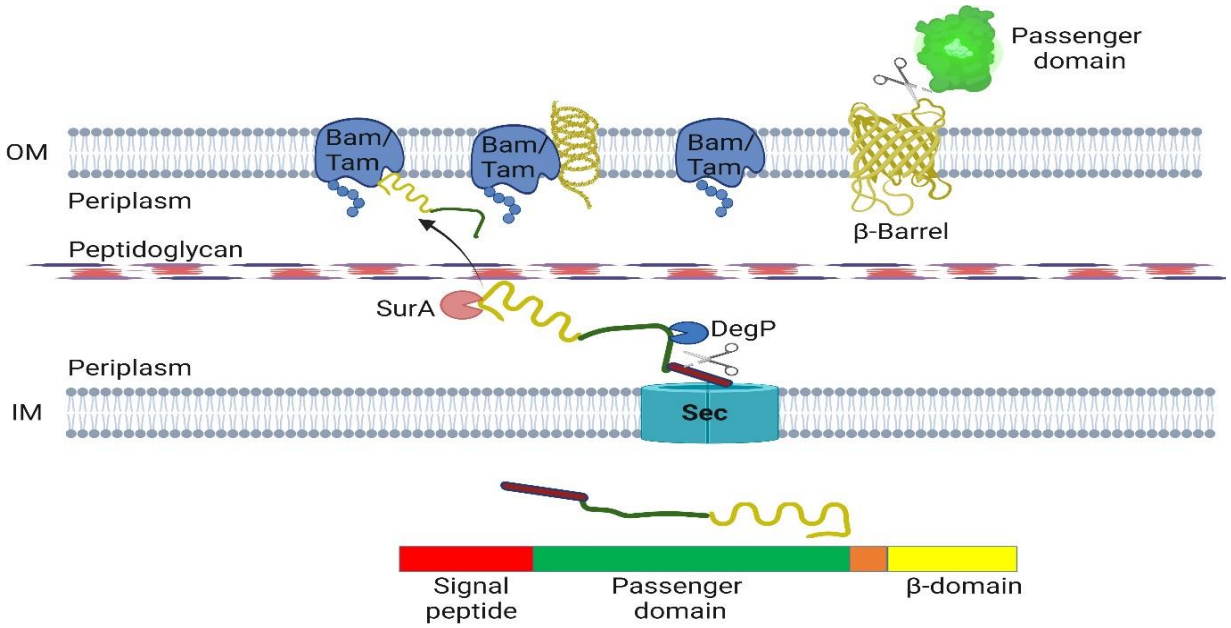

Figure 2.9 A Schematic representing the processing, export, and secretion of SAPTEs.

The Signal peptide assists the translocation of the autotransporter from cytoplasm to the periplasmic space via the Sec apparatus. In the periplasm, the passenger and β-domain are conserved in a "translocation competent state" and guided into Bam complex at outer membrane by chaperones such as SurA and DegP in unfolded state. The Bam complex helps in the integration of the β-domain into the outer membrane and promotes the formation of β-barrel to translocate of the passenger domain across the outer membrane via a hybrid-barrel mechanism. During this mechanism barrel expansion results in the opening of pore and the passenger domain can stick out through the hybrid barrel. Successively, the folded passenger domain is either cleaved and released from the bacterial cell or remains attached on the surface of the bacteria. (Adapted/modified from (Pokharel *et al.*, 2019)). The figure was created in Biorender.com.

iii) It has been reported that BamA and TamA are implicated in the translocation of autotransporter proteins from the outer membrane. BamA and TamA are homologues and share similar mechanisms of chaperone foldase and insertase activity. In addition, BamA is also known to promote the insertion and folding of the β-domain into the outer membrane, and a 'hybrid barrel' model has been proposed for the export of the folded passenger domain from the outer membrane (Sauri *et al.*, 2009). BamA/TamA barrels can incorporate the β-domain of the autotransporter protein by β-augmentation leading to the formation of a hybrid AT b β-barrel domain with BamA/TamA. This hybrid barrel contains a pore via which the AT passenger domain will be folded and translocated outside the outer membrane. Once, the autotransporter protein is translocated the hybrid β-domain barrel dissociates from the BamA/TamA: β-barrel complex releasing BamA/TamA complex into its uncoupled state (Gruss *et al.*, 2013; Selkrig *et al.*, 2012; Stubenrauch *et al.*, 2019).

iv) Most of the ATs from the SPATEs family such as Vat, Tsh, Sha, Pic, EspP and SepA are released into the extracellular environemnt via the outer membrane (Jacob-Dubuisson *et al.*, 2004), whereas some remain associated with the outer membrane of bacteria for example BrkA (Oliver *et al.*, 2003). Passenger domains of most of the SPATEs are cleaved by auto-catalytic activity inside the β-barrel and they have conserved cleavage sites. Cleavage of ATs is a crucial step for their release into the surroundings and takes place by several mechanisms (Dautin *et al.*, 2007).

2.10.2.2 Characteristics of cleavage site of SPATE proteins

The cleavage site of SPATE proteins is located and conserved in the linker domain. This linker domain contains a conserved 14 residue segment that connects the translocation and passenger domains. SPATEs such as EspP and Tsh are known to have a motif (^{1021}EVNNLNKRMGDL^{1032}) where the cleavage site of the passenger domain is after the first asparagine (twin asparagine) residue (Dautin *et al.*, 2011; Kostakioti *et al.*, 2006). Even though some SPATEs such as RpeA and Sha lacks twin asparagine residues, they are known to be released into the culture supernatant (Habouria *et al.*, 2019; Leyton *et al.*, 2007). This altogether suggests that linker domain of ATs plays a crucial role in the secretion of the passenger domain, however, motifs other than the twin asparagine motif are important for cleavage of the passenger domain of certain SPATEs.

2.10.3 Characteristics features of SPATE proteins.

As the name suggests, SPATE proteins are the family of autotransporter proteins of the enterobacterial species that possess a conserved serine protease motif. They have been described in different enterobacterial species such as pathogenic *Escherichia coli, Shigella spp., Citrobacter rodentium, Serratia marcescens, Salmonella bongori* and *Edwardsiella tarda.* Their specific features consist of the β-domain whose identity is conserved from 60-90% among different proteins, a consensus serine protease motif “GD**S**GSP” with a catalytic serine (**S)** within the N-terminal domain between amino acid residues 250-270, a passenger domain which is mostly secreted into the culture media, and a long (usually >50 amino acid) signal peptide that enables post-translational targeting during secretion (Pokharel *et al.*, 2019).

Structurally and functionally, SPATEs are classified into class 1 and class 2. The cytotoxic and cytopathic toxins that stimulate loss of membrane integrity, cytoplasmic shrinkage, and activation of apoptosis in host cells are grouped into class 1. SPATEs such as Sat, EspP, SigA, Pet, EspC, and

TagC belong to this class. Class 2 SPATEs are O-glycoproteases that cleave O-glycoproteins and mucin from host cells. SPATEs such as Tsh, Vat, EatA, Pic, PicU, SepA, Boa, EaaA, TagB, Sha, and TleA belong to this class. In addition, SPATEs can also trigger specific host immune responses (Dutta *et al.*, 2002; Pokharel *et al.*, 2019).

2.10.4 Distribution of SPATEs among the pathogenic *E. coli*

Some of the most characterized SPATEs are the vacuolating autotransporter toxin (**Vat**), temperature-sensitive hemagglutinin (**Tsh**), extracellular serine protease plasmid (pO157-encoded) from enterohemorrhagic *E. coli* (EHEC) (**EspP**), plasmid-encoded toxin (**Pet**), protein involved in intestinal colonization from EAEC and uropathogenic *E. coli* (UPEC) and *Shigella* (**Pic**), secreted autotransporter toxins from UPEC (**Sat**), EPEC secreted protein C (**EspC**), ETEC autotransporter A (**EatA**), two recently characterized serine-protease hemagglutinin autotransporter from APEC and UPEC strains (**Sha**) and tandem autotransporter genes B and C (**TagBC**). Among these, Tsh is present in NMEC and APEC strains; Vat, Sha and TagBC are present in APEC and UPEC strains; Sat is present in EAEC, UPEC, DAEC and *Shigella*; SepA is present in DEAC and *Shigella;* and SigA, EspC, EspP. EatA are exclusively present in *Shigella,* EPEC, EHEC and ETEC respectively (Habouria *et al.*, 2019; Parreira *et al.*, 2003; Pokharel *et al.*, 2019) (**Figure 2.10**).

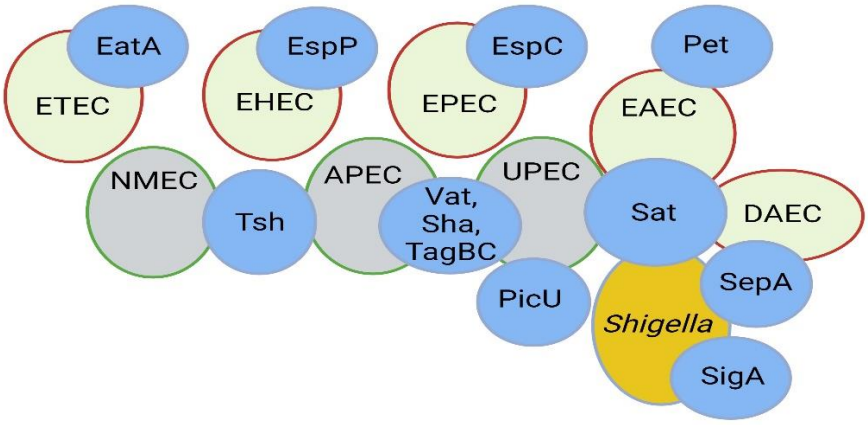

Figure 2.10 Distribution and overlap of popular SPATES among InPEC and ExPEC

SPATE proteins (depicted in blue circle) are distributed widely among InPEC (represented in pale green and red circle) and ExPEC (showcased in grey and green circle). Some of these SPATEs are commonly present in InPEC, ExPEC and even in *Shihella* spp. (APEC, Avian pathogenic *E. coli*; UPEC, Uropathogenic *E. coli*; NMEC, Neonatal meningitis *E. coli*; EPEC; Enteropathogenic *E. coli*; EAEC, Enteroaggregative *E. coli*; EHEC, Enterohemorrhagic

***E. coli*; ETEC, Enterotoxigenic *E. coli*; DAEC, Diffuse Adhering *E. coli*.) (Adapted/modified from (Pokharel *et al.*, 2019)). The figure was created in Biorender.com.**

2.10.5 SPATEs of avian pathogenic *E. coli* (APEC O1: K1)

From the complete genome sequence of APEC O1: K1 strain QT598, isolated from a 4-day old turkey, five different SPATEs were identified. Among these, two were previously characterized Vat and Tsh, and three were recently characterized by our research group - Sha, TagB, and TagC (Habouria *et al.*, 2019). Since strain QT598 can produce five SPATEs and the traits of both UPEC and APEC strains, this strain was chosen as a model strain to investigate the hierarchical or combined role of different SPATEs in a systemic infection model. Distinguished features of these five SPATEs are described below.

2.10.5.1 Tempreature-sensitive hemagglutinin (Tsh)

Tsh was the first characterized SPATE. It was identified in APEC strain χ7122 as a hemagglutinin of chicken erythrocytes encoded on a ColV-type plasmid. Tsh can agglutinate chicken erythrocytes in a mannose-resistant manner. However, hemagglutination activity was lost at higher temperatures. The production of Tsh was reportedly higher at lower temperatures (26 °C) in an *E. coli* K-12 background and the bacteria releases Tsh into the culture medium at higher temperatures (Dozois *et al.*, 2000; Pokharel *et al.*, 2019; Provence *et al.*, 1994). Tsh is a 1377 amino acid protein of ~148 kDa. The sequence contains the characteristic features of SPATEs i.e., a signal peptide (leader sequence) up to residues 53, a passenger domain containing a serine protease motif (S_{259}) extending from amino acid residues 53 to 1100, and a β-barrel domain extending from residues 1101 to 1377. The passenger domain is ~106 kDa and the β-barrel domain is ~33 kDa (Dozois *et al.*, 2000).

Since the amino acid sequence of Tsh is 77% identical to the Vat autotransporter protein, it is not surprising that there have been reports and genome sequence entries that have misnamed these two distinct SPATEs. For instance, the Vat protein in UPEC strain CFT073 and other UPEC strains were erroneously reported as Tsh (Heimer *et al.*, 2004). Later (Restieri *et al.*, 2007) identified the difference in distribution of Vat and Tsh. In addition to amino acid differences also that *tsh* is predominantly encoded on some ColV-like or similar virulence plasmids, whereas the *vat* gene is associated with pathogenicity islands and phylogenetically distributed and present in particular in members of the B2 clonal group (Restieri *et al.*, 2007).

Later it was characterized and shown to be present in human ExPEC strains of NMEC and APEC strains (Nicholson *et al.*, 2016; Provence *et al.*, 1994). A Tsh-like protein that shares 60 % identity and different biological functions was also reported in the fish pathogen, *Edwardsiella tarda.* Tsh is also shown to share some features of other serine-protease autotransporters including IgA proteases from *Hemophilus influenzae and Neisseria gonorrhoea* with 56% similarity. However, Tsh does not have the ability to cleave IgA (Hu *et al.*, 2016; Stathopoulos *et al.*, 1999). Hemoglobin protease (Hbp) is a near-identical variant of Tsh, which differs at only two amino acid positions (Q209K and A842T). Hbp was isolated from a patient with a wound is encoded on a ColV plasmid pColV-k30 , was known to cleave hemoglobin (Otto *et al.*, 1998) and was shown to contribute to heme-dependent growth of *Bacteroides fragilis* in an abscess co-infection model with *E. coli* strain EB1 (Luirink *et al.*, 2002).

Tsh can agglutinate erythrocytes from several species including humans, sheep, dogs, horses, turkeys, pigs, cattle, and rabbits. As such it may promote virulence in multiple host species. Moreover, Tsh has been shown to promote adherence to Caco-2 cells and extracellular matrix proteins such as collagen IV, laminin, and fibronectin (Kostakioti *et al.*, 2004). Tsh was also shown to numerous host proteins including factor V, mucin, and O-glycosylated proteins such as CD43, CD44, CD45, CD93, CD162, and CX3CL1 in vitro (Ayala-Lujan *et al.*, 2014; Ruiz-Perez *et al.*, 2011). Tsh also contributes to the APEC infection in the chicken respiratory tract by promoting the progression of infection and development of lesions and deposition of fibrin in air-sacs (Dozois *et al.*, 2000). Tsh has also demonstrated enterotoxin activity in a rabbit ileal loop model (Maluta *et al.*, 2014a). Altogether, Tsh has various roles in pathogenesis of APEC and other ExPEC along with cellular and immune modulation functions (Dutta *et al.*, 2002; Habouria *et al.*, 2019; Kostakioti *et al.*, 2004; Pokharel *et al.*, 2019).

2.10.5.2 Vacuolating autotransporter toxin (Vat)

Vacuolating autotransporter toxin (Vat) belongs to class II SPATE group and was originally identified as ORF27 from a pathogenicity island present in Avian pathogenic *E. coli* (APEC), strain Ec222 (Parreira *et al.*, 2003). The gene encoding for Vat is situated in between *proA* and *yagU* genes on the pathogenicity island (VAT-PAI). Vat from Ec222 also contains characteristic features of SPATEs wherein the 1376 amino acid precursor protein contains a 55 amino acid N-terminal signal sequence, a passenger domain containing a serine protease motif (S_{260}), and a C-terminal outer membrane translocator β-barrel domain (Parreira *et al.*, 2003).

As the name suggests the cytotoxicity phenotype of Vat was defined from the observation of the formation of vacuoles in chicken embryonic fibroblast (CEF) cells from 2h-24 h after exposure. Vat has been shown to be an important virulence factor in the pathogenicity of avian septicemic *E. coli* (Ec222) and other APEC strains. Vat was shown to have a crucial role in the respiratory tract infection, cellulitis, and septicemia in poultry. Chickens challenged with Ec222 in a cellulitis infection model, the vat mutant infected chickens showed inability to develop cellulitis compared to wildtype strain (Parreira *et al.*, 2003; Pokharel *et al.*, 2019). In addition, Vat also contributed to the fitness of UPEC strains during systemic infection of murine and highly conserved in urosepsis clinical isolates (Nichols *et al.*, 2016). Vat is expressed in the murine urinary tract (Heimer *et al.*, 2004) and in urosepsis patients there is a high titer of Vat-specific IgG in the plasma of patients infected with *vat*-positive UPEC strains compared to patients with *vat*-negative UPEC strains (Nichols *et al.*, 2016). Moreover, Vat was also shown to induce non-lysosomal vacuole formation; depletion of stress fibers and damage of F-actin distribution; dysregulation of the urothelial barrier of bladder epithelial cells (Díaz *et al.*, 2020). In a murine UTI model, Vat along with other SPATEs contributed to fitness in colonization and cytotoxicity of kidneys by UPEC strains (Subashchandrabose *et al.*, 2013). Furthermore, a recent study in mice model suggested that Vat and Tsh were significantly upregulated and played a crucial role in the pathogenicity of ExPEC strain PU-1 during bloodstream infection (Pan *et al.*, 2023). In addition to ExPEC strains, a variant of Vat was isolated from a patient with AIEC related Crohn's disease. This Vat-AIEC showed around 97% similarity with Vat-Ec222 with minor modifications in the catalytic domain (Gibold *et al.*, 2016). Altogether Vat is a crucial virulence factor during the pathogenesis of UPEC and APEC strains.

2.10.5.3 Serine-protease hemagglutinin autotransporter (Sha)

The Serine-protease hemagglutinin autotransporter (Sha) is a recently identified class II SPATE that is encoded by a large ColV-type plasmid pEC598 of APEC strain QT598. The *sha* gene is adjacent to the genes encoding a P-like fimbrial (*plf)* gene cluster (Habouria *et al.*, 2022). Although some APEC and UPEC strains contain *sha* gene sequences, *sha* was more common in APEC strains. Compared to other SPATEs, Sha shares 43% identity with Tsh and 38% identity to Vat. Sha also shares similar characteristic features of SPATEs including a long N-terminal signal peptide, passenger domain and translocator domain. Sha is a 1302 amino acid precursor protein with a serine protease motif at S_{258} position.

Functionally Sha was shown to cleave N-Succinyl-Ala-Ala-Ala-p-nitroanilide signifying its elastase activity; increased adherence to avian and human epithelial cells when cloned into non-

adherent *E. coli* K-12 strain; and also demonstrated hemagglutinating activity for turkey, human, chicken, sheep, bovine, rabbit, horse, pig and dog erythrocytes (Habouria *et al.*, 2019; Pokharel *et al.*, 2019). In a murine UTI model, expression of *sha* was shown to be upregulated six-fold in bladder, however, its role in competitive fitness during colonization of the urinary tract by bacteria was not demonstrated (Habouria *et al.*, 2019). Currently, Sha has been investigated less compared to other SPATEs although it may contribute collectively to pathogenesis of some APEC and UPEC strains.

2.10.5.4 Tandem autotransporter genes, *tagB* and *tagC* (TagBC)

The tandem autotransporter genes, *tagB* and *tagC* (TagBC) were recently identified SPATEs. TagB is a class II SPATE and TagC falls into class I subtype. As the name indicates the genes encoding for TagB and TagC SPATEs are adjacent to each other on a genomic island located between the conserved *yjdI* and *yjdK* genes of *Escherichia coli* in APEC strain QT598. TagB is a 1278 amino acids pre-protein with a serine protease motif at S_{250} whereas TagC is a 1322 amino acid precursor with a serine protease motif at S_{265} position (Habouria *et al.*, 2019; Pokharel *et al.*, 2019).

TagB and TagC encoding genes have been identified in both APEC and UPEC strains. Interestingly, APEC strains containing *tagBC* were exclusively isolated from turkey samples. TagBC were shown to cleave N-Benzoyl L-arginine 4-nitroanilide and demonstrate trypsin-like activity. Like Tsh and Sha they were also shown to confer hemagglutination and auto-aggregation activity. Tag B and TagC also promoted adherence to 5637 bladder cell lines and HEK 293 renal cell lines and cytotoxicity to human bladder cell lines when expressed in *E. coli* K-12. Regardless of these phenotypes, the loss of *tagB* and *tagC* did not change virulence or fitness in a murine UTI model (Habouria *et al.*, 2019; Pokharel *et al.*, 2019). However, their role in the pathogenesis of APEC and their ability to cause systemic infection via respiratory tract in poultry have not been investigated.

2.10.6 Regulation of expression of SPATEs

Although the roles of several SPATEs in pathogenesis in various infection models has been well established, aspects of regulation of expression of SPATEs have not been well characterized. Expression of SPATEs has been compared by investigating transcription levels under different growth conditions in vitro or in vivo, but regulatory mechanisms remain largely unknown. SPATEs such as SepA, Pic, EspC, and Tsh were shown to be regulated by temperature (Dozois *et al.*, 2000; Elisa Drago-Serrano *et al.*, 2006; Stathopoulos *et al.*, 1999). Expression of Tsh and Vat was also

shown to be upregulated in minimal medium compared to rich medium (Habouria *et al.*, 2019). However, the exact regulatory pathways for these SPATEs are yet to be discovered.

SPATE-encoding genes present on pathogenicity islands were assumed to be regulated by the locus of enterocyte effacement (LEE) encoded regulator (Ler). Genes present in LEE pathogenicity island were known to be positively regulated by Ler. Hence, it would be exciting to study their roles in production of SPATEs and their ability to induce virulence in various conditions (Elliott *et al.*, 2000).

One of the best examples of demonstration of regulation of SPATE gene expression was determined for the Vat autotransporter by the HN-S (**H**istone-like **n**ucleoid **s**tructuring) regulatory protein. H-NS is one of the universal regulators that can play an important role in bacterial gene regulation by condensing and super-coiling DNA (Dorman, 2004). The expression of *vat* in addition to other AT encoding genes such as trimeric autotransporters were shown to be regulated by H-NS (Pokharel *et al.*, 2019; Totsika *et al.*, 2012). In UPEC strain CFT073 strain, H-NS was shown to negatively regulate the expression of *vat* (Nichols *et al.*, 2016). In the same line, promoters of various autotransporters were shown to have putative H-NS binding sites (Pokharel *et al.*, 2019). These binding sites for APEC/UPEC SPATEs were listed in **table 2.8**. The presence of these sites signifies the potential role of H-NS in the regulation of expression of SPATEs, however, experimental evidence for such roles has not been elucidated. Another potential regulator of expression of *vat* is a gene present downstream of *vat* called *vatX*. VatX is a predicted regulatory protein belonging to the Multiple Antibiotic Resistance Regulator (MarR) family and contains a predicted DNA binding domain. In UPEC strain CFT073, VatX was shown to positively regulate the expression of *vat.* This suggests that VatX could potentially compete with H-NS to regulate the expression of *vat* (Nichols *et al.*, 2016; Pokharel *et al.*, 2019).

Another global transcription factor, the cyclic AMP receptor protein (CRP) was shown to effect regulation of the gene encoding the *pet* autotransporter, and its expression was shown to be co-regulated by CRP and the regulatory protein Fis (factor for inversion stimulation) .Further, other autotransporters including *sat* in UPEC and *sigA* in *Shigella sonnei* were shown to be co- regulated by Fis and CRP (Rossiter *et al.*, 2011; Rossiter *et al.*, 2015). Therefore, the possible role of these regulators could have a great potential in understanding the expression of different SPATEs and affect their role in *E. coli* biology and pathogenesis.

Table 2.8 Putative H-NS binding sites of promoters of APEC/UPEC SPATEs

SPATEs	Potential H-NS binding sites
tsh	-164CACATAAAGT-155 (-), -28AAAATAAAAT-19 (-), -10GTAATTAAAA-1 (+)
vat	-296TCCATATATC-287 (+), -295TGGATATATG-286 (-), -107GCTATATAAT-98 (-)
sha	-187CCCACAAATC-178 (-), -48TCCTTATATT-39 (+), -32TCAATAGATA-23 (-)
tagB	304ACGAAAAAAA-295 (-), -161CTGATAAATA-152 (-), -128TCGATAAATG-119 (+)
tagC	-256GCAATTAATA-247 (+), -62TCGCTATATT-53 (+), -56ACTATAAATA-47 (-)

This thesis is focused on the investigation of the role of 5 distinct SPATEs (serine protease autotransporters of *Enterobacteriaceae*) for the systemic infection and colonization in an air-sac infection model in young turkeys. Recently, our group reported the presence of five SPATEs in APEC strain QT598. Among them TagB, TagC and Vat are chromosomally encoded and Tsh and Sha are located on a ColV-type plasmid (pEC598-1). As detailed above these five SPATEs have different mechanisms of action, may recognize different host substrates, and demonstrate distinct types of toxicity as well as differences in regulation of expression in various host niches. Currently, the specific roles of these different SPATEs during infection in a poultry model have not been determined. Depending on the tissue environment and adaptability in host, one SPATE among these or in combination with another might contribute to establishment of disease or promote systemic infection. The detail of the research is presented in chapter 4, the Research Article chapter.

3 OBJECTIVES

As mentioned earlier, ExPEC are associated with variety of diseases in multiple hosts such as human, livestocks, and poultry. With the identification of different types of SPATEs in the APEC strain QT598, we could speculate that they could have specific roles during respiratory tract infection in a poultry model. Therefore, we hypothesized that as per the tissue environment and the ability of bacteria to adapt in a host, these SPATE proteins individually or in combination with one another could promote the establishment of disease or boost systemic infection. To test the hypothesis, the main objective of this thesis was to establish the hierarchical role of SPATEs during infection caused by an APEC, QT598 strain. For this purpose, we developed the following objectives.

i) To establish the turkey air-sac infection model.

To study the roles of these different SPATEs, various mutants including a quintuple (5x) mutant (Δ5SPATEs) were created and previously studied in a murine ascending urinary tract infection model (Habouria *et al.*, 2019). Deletion of all 5 SPATEs attenuated the strain in the kidneys whereas deletion of either *sha* or *tagBC* did not have any appreciable effect on colonization of the urinary tract. Since QT598 is of avian origin, we initially tested this strain in an established air sac infection model in chickens. However, QT598 which was originally isolated from a 4-day-old turkey poult, was not able to readily cause systemic disease in either a 5-day old or a 21-day old chicken infection model. As the strain was initially isolated from a diseased turkey, we considered that there could be potentially host-specific adaptation of the strain to turkeys. We therefore tested the capacity of strain QT598 as well as mutant derivatives that were missing different combinations of the SPATE genes in a 1-week-old turkey poult model. Interestingly, the Δ5SPATEs mutant was attenuated compared to the WT parent strain QT598. QT598 was able to colonize lungs, spleen, and liver, whereas the Δ5SPATEs mutant was much reduced in all these organs.

ii) To understand the regulatory hierarchy of SPATE system during the systemic infection of a host.

Although SPATEs collectively contribute to systemic infection in the turkey model, it is unknown whether any of these SPATEs may play a specific role during infection of host. To investigate this question, different SPATEs genes were re-introduced in single copy into the Δ5SPATEs mutant, with the complemented strains, tested for their capacity to colonize systemic tissues in the turkey. The single-copy complementation of SPATE-encoding genes was achieved using functional copies of

SPATEs expressed from their native promoters that were integrated/transposed into the *attTn7* site on the bacterial chromosome or by re-introduction of SPATEs genes into the non-functional *iucC* gene located on the pEC598-1 virulence plasmid. For this purpose, we generated single-copy *vat, tsh, sha, tagBC, vat-tagBC, sha-tsh* and quintuple (5x) complemented strains. The potential role of different SPATEs for avian respiratory infection was then studied by performing the air-sac infection in turkeys using these strains and by investigating gene expression of SPATEs in different mutants. Altogether, the major objective of the research is to establish the hierarchical role of SPATEs in strain QT598 of *E. coli*.

4 RESEARCH ARTICLE

4.1 Title of manuscript and authors contribution

The plasmid-encoded serine protease autotransporters Tsh and Sha contribute to avian pathogenic *Escherichia coli* colonization of the lungs in turkey.

(Les autotransporteurs de sérine protéase Tsh et Sha, codés par un plasmide de virulence, contribue à la capacité d'une souche d'*Escherichia coli* pathogène aviaire d'infecter des poumons chez la dinde.)

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Status: Ready to be submitted

Targeted journal: Virulence (Taylor & Francis)

Contribution of authors:

Conceptuawastion and visualization, **S.D., and P.P**.; formal analysis, **S.D., and P.P.;** and supervised by **C.M.D.** Writing — original draft preparation, **S.D., and P.P**. Cloning, molecular biology experiments (including primer designing); protein extraction for SDS-PAGE, western and Mass Spectrometry; western, SDS-PAGE performed and the respective sections in manuscript were written by **S.D**. Phenotypic analysis of complemented strains such as auto aggregation, proteolytic cleavage assay were performed and respective sections in manuscript were written by **P.P**. Turkey infection and subsequent analysis, qPCR, and mass spectrometry analysis were performed by **S.D**., and **P.P. S.H.** was continuously supervising while experiments were being performed and rigorously involved during the turkey infection studies. The manuscript was reviewed and edited by**, S.D., P.P., S.H., and C.M.D**. Funding acquisition, **S.D., P.P., and C.M.D.**

4.2 Abstract

Serine protease autotransporters of Enterobacteriaceae (SPATEs) are high molecular weight secreted proteins that contribute to virulence and function as proteases, toxins, adhesins, and/or immunomodulators. These virulence factors can play an important role in the different stages of bacterial infection of different host species. An extra-intestinal pathogenic *E. coli* (ExPEC) O1:K1 strain, QT598, originally isolated from a turkey, was shown to produce five distinct types of SPATE proteins: Tsh, Sha, Vat, TagB, and TagC. Herein, we investigated the cumulative and specific roles of SPATEs in a turkey respiratory infection model. In order to do this, different combinations of SPATE-encoding genes were re-introduced into the genome of a mutant derivative of QT598 lacking all 5 SPATEs genes (Δ5SPATEs) in the turkey model of infection. Loss of all 5 SPATEs resulted in a significant reduction in colonization in extraintestinal tissues including lung and liver. Complementation of the Δ5SPATEs mutant with all 5 SPATE–encoding genes restored bacterial numbers to wild-type parent QT598 levels. Interestingly, complementation with only the *tsh* and *sha* genes was sufficient to restore levels of infection in the lungs to wild-type levels. By contrast, all five SPATE genes were found to be expressed *in vivo* in infected turkey lungs and air sacs, however expression of *tsh* and *sha* genes was markedly higher than expression of the other SPATEs in these respiratory tissues. Taken together, results demonstrate an important role for SPATEs for *E. coli* infection of turkey tissues, further, the *tsh* and *sha* genes encoded on a ColV-type virulence plasmid pEC598 play a predominant role in infection of the turkey lungs.

4.3 Introduction

Avian pathogenic *Escherichia coli* (APEC), a subset of extra-intestinal pathogenic *Escherichia coli* (ExPEC) is responsible for a wide range of localized and systemic infections in poultry known as avian colibacillosis; that includes infection of fowl including chickens, ducks, turkeys and other avian species (Dho-Moulin *et al.*, 1999). Avian colibacillosis is responsible for the mortality, morbidity and/or reduced productivity of affected birds costing the poultry industry hundreds of millions of dollar in economic losses worldwide (Nolan *et al.*, 2013). APEC can cause swollen head syndrome, air sacculitis, perihepatitis, pericarditis, salpingitis, egg peritonitis, cellulitis, and osteomyelitis (Nolan *et al.*, 2013) in birds. *E. coli* belonging to a variety of different serogroups have been associated with field outbreaks, however three serogroups - O1, O2 and O78 have often been more commonly associated with APEC strains (Ghunaim *et al.*, 2014).

Colibacillosis can occur following entry of APEC either through the respiratory or oral route and strains can either be primary or secondary pathogens if other infectious agents (viruses mycoplasma, coccidian infections) or environmental stresses predispose birds to subsequent APEC infection (Dho-Moulin *et al.*, 1999). After colonization of trachea, lungs and air sacs, APEC can disseminate into the circulatory system and cause systemic infections– including pericarditis, perihepatitis and bacteremia (Dho-Moulin *et al.*, 1999; Dziva *et al.*, 2008).

In addition to causing infections in poultry, the question of whether some APEC strains may have a foodborne zoonotic potential have been raised, since reports have demonstrated that some APEC isolates (belonging to sequence types ST95, ST131 or O1, O2, and O18 serotypes) from infected poultry or isolated from poultry meat products are closely related to human ExPEC strains isolated from extraintestinal infections such as urinary tract infections, sepsis and neonatal meningitis (Mellata, 2013; Moulin-Schouleur *et al.*, 2006; Nandanwar *et al.*, 2014). Further, virulence plasmids such as ColV (colicin V)-plasmids that are typically present in nearly all APEC strains can also be found in certain ExPEC isolates from human infections (Liu *et al.*, 2018).

APEC strains contain a variety of virulence genes that mediate colonization of host tissues and survival evasion of host immune defenses. These factors include adhesins, invasins, toxins, iron acquisition systems and protectins (Dho-Moulin *et al.*, 1999; Dziva *et al.*, 2008). Among some of the virulence factors described for APEC some autotransporter proteins have been identified including the SPATEs (serine protease autotransporters of *Enterobacteriaceae*) Vat and Tsh. SPATEs are high molecular weight proteases secreted by APEC and are known to have multiple functions – such

as adherence, cytotoxicity, and modulation of host immune functions (reviewed in (Clarke *et al.*, 2022; Dautin, 2010; Kathayat *et al.*, 2021; Pokharel *et al.*, 2019)).

Our group identified five distinct SPATEs that were present in APEC O1:K1 strain, QT598 (Habouria *et al.*, 2019), which was originally isolated from 4-day old turkey poult. Among these SPATEs two SPATES Tsh and Vat had been described and were known to contribute to infection in either chicken or murine models of extra-intestinal infection. In addition, three new SPATEs Sha, TagB and TagC were identified for the first time. In a previous study we investigated the biological activities of these SPATEs and their cumulative roles in a murine ascending urinary tract infection (UTI) model (Habouria *et al.*, 2019).

Herein, we investigated the role of the different SPATEs for APEC strain QT598 in a turkey respiratory infection model and demonstrate their hierarchical importance for colonization/infection of specific tissues in young turkey poults.

4.4 Materials and methods

4.4.1 Ethics Statement

This study was performed in accordance with the ethical standards of the INRS campus and the National Experimental Biology Laboratory at INRS. The protocol for airsac infection of turkeys was approved by the animal ethics evaluation committee – Comité Institutionel de Protection des Animaux (CIPA No 1908–02) of the INRS-Armand-Frappier Santé Biotechnologie Research Centre.

4.4.2 Bacterial strains, Plasmids, and Growth Conditions

All strains, plasmids and primers are listed in table 4.1-4.2. APEC strain QT598 and its derivative strains were grown at 37 °C and QT6043 was grown at 30 °C on solid or liquid Luria-Broth medium (Alpha Bioscience, Baltimore, MD, USA). The antibiotics were supplemented when required with concentrations of 30 µg/mL chloramphenicol, 100 µg/mL ampicillin, 15 µg/mL of gentamycin, or 50 µg/mL of kanamycin. Modified M9 glycerol medium was prepared with following compositions: (1X) M9 salts 6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl with 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, 0.2% glycerol, 15 mg/ml proline, 3 mg/ml thiamine. For turkey infection studies, QT598, its mutants and complemented derivatives were grown in brain heart infusion broth (Alpha Bioscience, Baltimore, MD, USA).

4.4.3 Generation of vectors to complement the APEC SPATE deletion-mutant with different SPATE-encoding genes.

Cloning of *vat, tagBC, tsh,* and *sha* genes was performed by PCR amplification of DNA segments including the native promoter regions of each of the genes from genomic DNA of strain QT598, using specific primers (**Table 4.2**) and Phusion flash high-fidelity PCR master mix (Thermo Fisher Scientific, Waltham, MA, USA). The inserts obtained from PCR amplification contained Gibson homologies to the multi-cloning site (MCS) of the mini-Tn7 containing plasmids to the *StuI* site of pGP_miniTn7_Gm for *vat* and *sha;* and homologies to the *XhoI* and *StuI* sites of pGP_miniTn7_Gm for *tagBC* and *tsh.* DNA segments for *vat* and *sha* were assembled using the pEASY-UNI assembly kit (TransGen Biotech, Beijing, China) to pGP_miniTn7_Gm linearized by restriction enzyme *StuI* (New England BioLabs, Ipswich, MA, USA) to obtain plasmids pIJ615 and pIJ618. Inserts for *tagBC* and *tsh* were assembled using the pEASY-UNI assembly kit to pGP_miniTn7_Gm linearized by restriction enzymes *XhoI* and *StuI* (New England BioLabs, Ipswich, MA, USA) to obtain plasmids pIJ616 and pIJ619. Further, cloning of *vat* and *sha* genes into pIJ616 and pIJ619 respectively were performed by PCR amplification using specific primers (**Table 4.2**) from genomic DNA of QT598. Inserts of *vat* and *sha* were assembled using the pEASY-UNI assembly kit to pIJ616 and pIJ619 linearized by restriction enzyme *XhoI* (New England BioLabs, Ipswich, MA, USA) to obtain plasmids pIJ617 and pIJ620 respectively.

Cloning of *sha* and *tsh* were performed by PCR amplification using specific primers (**Table 4.2**) and Phusion flash high-fidelity PCR master mix that includes the gentamycin resistance cassette and promoter from plasmid pIJ620, the insert was then assembled into the MCS of vector pIJ266, pBluescript II SK (+) derivative containing Km^r cassette between fragments of the *lacZ* and *lacA* genes, linearized using *KpnI* and *XhoI* (NEB) using the pEASY-UNI assembly kit. This generated plasmid pIJ622 was then used as a template to amplify the *sha-tsh* genes using primers containing flanking regions homologous to *lacZ* and *lacA* region (*lacZ'-kan-Gm-sha-tsh-lacA'*) of QT598 genome and Gibson region homologous to MCS of suicide plasmid pMEG-375 digested with *PacI* and *PmeI*. The inserts were assembled into pMEG-375 linearized with *PacI* and *PmeI* (NEB) using the pEASY-UNI assembly to obtain plasmid pIJ623. This plasmid was used to integrate the *sha-tsh* gene into the *lac* region of QT598 by *sacB*-mediated allelic-exchange as described in (Porcheron *et al.*, 2014).

4.4.4 Protein Preparation and Western Blot Analysis

Overnight cultures in a 5 ml volume were grown for each of the SPATE-encoding clones within a DH5α λ-*pir* strain background: *vat* (QT6023), *tagBC* (QT6024), *vat-tagBC* (QT6025), *sha* (QT6026), *tsh* (QT6027), and *sha-tsh* (QT6028) to allow medium-copy replication of the pGP_miniTn7_Gm plasmid that was used to clone the SPATEs.

Supernatants were separated by centrifugation at 6000 x *g* for 15 minutes at 4 °C. Supernatants were then incubated in 15% trichloroacetic acid (TCA) (v/v) to precipitate SPATE proteins. The precipitated proteins were then concentrated using centrifugation at 14000 x *g* for 20 min at 4°C. These proteins were then washed consecutively with Tris-EDTA (0.05 M) pH 12 then Tris-EDTA (0.05 M) pH 8.5; and resuspended in 0.2 ml of Tris-EDTA (0.05 M) pH 8.5 as described in (Habouria *et al.*, 2022). SDS-PAGE loading samples (1x) were prepared using the 4x Laemmli sample buffer (Tris-HCL 200 mM, 8% SDS (v/v), 40% glycerol (v/v), 4% β-mercaptoethanol (v/v), 50 mM EDTA, and 0.4% bromophenol blue (v/v)) and resolved in 10% SDS-PAGE gel at 200 V for 40 min and stained with Coomassie blue to visualize protein bands as described in (Habouria *et al.*, 2019).

Western blot analysis was performed as previously described (Crépin *et al.*, 2012b) with some modifications. For Western blotting analysis proteins were extracted from SPATE-complemented strains *vat* (QT6044), *tagBC* (QT6045), *vat-tagBC* (QT6046), *tsh* (QT6047), *sha* (QT6048), *sha-tsh* (QT6051), WT (parent strain QT598), *mutant* (Δ5SPATEs mutant QT6042), 5x complementation (5 SPATEs complemented mutant strain QT6052), where supernatants from 50 ml overnight cultures were filtered and concentrated through 50 kDa cut-off Amicon filters and concentrations were measured using the Pierce™ Coomassie Plus (Bradford) Assay Reagent (Thermo Fisher Scientific). Loading samples were prepared, and 10 µg of proteins were migrated and resolved on 10% SDS-PAGE gels. Proteins were then electroblotted to nitrocellulose membrane (Pall corporation, Port Washington, USA). The membrane was blocked in tris-buffered saline (TBS) with 0.1% Tween-20 (TBST) and 5% (w/v) skim milk for 1 hour at room temperature. The membrane was then incubated overnight at 4 °C with Vat-specific rabbit polyclonal antibody (Pokharel *et al.*, 2020) diluted (1:2500), in TBST containing 5% skim-milk. This antibody cross-reacts with all of the SPATEs proteins due to conserved epitopes present in the proteins. The membrane was then washed three times using TBST and incubated with secondary antibody anti-Rb HRP diluted (1:10000) (Novus), in TBST for one hour at room temperature and washed three times using TBST. Signals were then revealed using SuperSignal™ West Pico PLUS Chemiluminescent Substrate

(Thermo Fisher Scientific, Waltham, MA, USA) and ChemiGenius 2 documentation system according to manufacturer's instruction.

4.4.5 Autoaggregation Test

The autoaggregation test was carried out as described in (Habouria *et al.*, 2019). Briefly, overnight cultures of the different strains were adjusted to an OD_{600} of 1.5. In the sterile glass tube 10 ml of each culture was transferred. Tubes were then vortexed for 5 s then allowed to stand statically at 4 °C for 3 h. The rate of autoaggregation was assessed by measuring the decrease in the OD_{600} of turbidity of the cultures from 1 cm deep from the top of the tubes at 3 h interval. The reduction in turbidity is then plotted as the value of the optical density. The autoaggregation percentage was expressed as $[(1 – (OD_{3h}/OD_{0h})) \times 100]$ where OD_{3h} represents the optical density (OD) of the culture at the incubation time of 3 h, while OD_{0h} is the OD of the culture at time 0 h.

4.4.6 Oligopeptide Cleavage Assays

Serine protease activity on the cleavage of synthetic peptides was performed as explained in (Dutta *et al.*, 2002). Briefly, 5 µg/ml of each SPATE-containing supernatant from different complemented strains in QT598 Δ5SPATEs background were added separately to 200 µl of three different pNA-conjugated oligopeptides: N-Succinyl-Ala-Ala-Ala-p-nitroanilide, N-Benzoyl-L-arginine 4-nitroanilide and N-succinyl-ala-ala-pro-phe-p-nitroanilide (Millipore Sigma, Burlington, USA) at 1 mM concentration in a buffer containing 0.2 M NaCl, 0.01 mM ZnSO4, 0.1 M MOPS (3-(N-morpholino) propane sulfonic acid) pH 7.3, in 96-well microtiter plates. The hydrolysis of these synthetic chromogenic substrates by the tested SPATEs was assayed at 37 °C. After 3 h of incubation, end-point fluorescence of the released p-nitroaniline was monitored at 410 nm. Readings were normalized to the maximum absorbance of positive control. All reactions were performed in triplicate. All reactions were performed in triplicate, and the mean ± SD was calculated.

4.4.7 Airsac Infection Model of Turkeys

1-day old turkey poults of both sexes in equal number were purchased from a local hatchery and transported to the animal facilities at the National Experimental Biology Laboratory at INRS-AFSB campus in Laval, Quebec. All experiments were conducted under the "Comité Institutionnel de Protection des Animaux" approved CIPA protocol number (CIPA No 1908–02). The turkeys were separated in different isolators with a maximum of 10 in each study group. On day 6, the turkeys were challenged directly in the left thoracic air sac with either wild-type strain QT598, QT598

Δ5SPATEs, and different strains wherein different combination of SPATE-encoding genes were re-introduced into the genome of the Δ5SPATEs mutant. The infectious dose was 3×10^8 CFU administered in a 100 ml volume. Turkeys in all groups were monitored daily after challenge for any clinical symptoms (behavior, posture, eyes opening, and respiratory distress signs) until 48 h. Necropsies were performed 48 h after challenge to examine lesions in the air sacs, heart, and liver. The macroscopic fibrinous lesions were examined, and lesion scores were recorded. The right airsac, right lung, spleen, and liver were harvested, weighed, and homogenized to determine bacterial numbers and also used for RNA extraction. Serial dilutions of samples diluted in sterile BSG containing NaCl 8.5 g/l, KH_2PO_4 0.3 g/l, Na_2HPO_4 0.6g/l, gelatin 0.1 g/l. The diluted samples were plated on MacConkey-lactose agar plates that were incubated for 16 to 18 hours at 37 °C. Bacterial numbers in the blood were determined by bacterial counts from blood samples taken at 6 and 48 hours after the challenge.

4.4.8 qRT-PCR Analysis of SPATE gene expression *in vivo* and *in vitro*

The expression of SPATE-encoding genes was determined by analysing RNA transcript levels of the 5 SPATE genes after growth in different culture media (LB medium, minimal M9 glycerol medium) and in vivo from infected turkey tissue samples. Bacterial cultures were grown in quadruplicate in LB broth (Alpha Bioscience) and M9-glycerol minimal at 37 °C. RNAprotect (Qiagen) was added to bacterial cultures and RNA was extracted using the EZ-10 Spin Column Total RNA Miniprep Kit (BioBasic) according to the manufacturer's protocol. To eliminate contaminating DNA from RNA samples, Ambion Turbo DNase (Thermo Fisher Scientific) was used, and the eluted RNA samples were confirmed by the detection of *rpoD* gene by PCR (40 cycles). For *in vivo* analysis, turkey air sac and lung samples were taken from 4 random animals infected with wildtype strain, QT598. The tissues were then homogenized with TRIzol® LS reagent (Thermo Fisher Scientific), centrifuged for 30 sec at 12,000 × *g*, and the supernatant was incubated with ethanol (95-100%) and transferred into Zymo-Spin™ IICR Columns (Zymo Research). RNA concentrations and integrity were determined by A260/A280 Nanodrop readings and agarose gel electrophoresis, respectively. Total RNAs were then reverse transcribed to cDNAs using *TransScript*® all-in-one first-strand cDNA synthesis supermix kit (TransGen Biotech Co., Ltd, Beijing, China) according to the manufacturer. Specific primers (**Table 4.2**) against each of the five different SPATE-encoding genes were used. The RNA polymerase sigma factor *rpoD* was used as a housekeeping control. qRT-PCR was performed in the Corbett Rotorgene (Thermo Fisher) instrument with reaction mixtures containing 50 ng of cDNA, 100 nM of each primer and 10µl of *TransStart*® tip green qPCR supermix (TransGen

Biotech Co., Ltd, Beijing, China). Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Livak *et al.*, 2001). Genes with a fold-change above threshold of 2 were considered as differentially expressed.

4.4.9 Mass Spectrometry for Peptide Analysis of SPATE proteins from culture supernatants

For mass spectrometry analysis, the proteins were extracted from the parental APEC strain QT598 and from different strains wherein certain SPATE-encoding genes had been deleted: Δ*tsh*; Δ*sha*; Δ*tagBC*; Δ*tagBC* Δ*vat*; Δ*tsh* Δ*sha*; Δ*tagBC* Δ*va t*Δ*sha*; and Δ*tagBC* Δ*vat* Δ*tsh*. For each of the samples, supernatants were obtained by centrifugation at 6000 x *g* for 30 min from 200 ml overnight cultures grown at 41 °C, shaking at 250 rpm (OD_{600} = 0.9-0.95).

The supernatants were then concentrated by size exclusion through 50 kDa cut-off Amicon filters at 2500 x *g*. SDS-PAGE was run as explained above and the protein bands at the 100 kDa range in the gel were excised and sent for mass spectrometry analysis at the proteomics platform of the Institut de Recherche en Immunologie et en Cancérologie (IRIC) (Montreal, Quebec, Canada). Data were analyzed and processed using Scaffold (version Scaffold_5.2.1, Proteome Software Inc., Portland, OR) to validate MS/MS-based peptide and protein identifications. Using Percolator posterior error probability calculation, protein were identified if they established more than 90.0% probability (Käll *et al.*, 2008). If the identified protein could establish more than 95.0% probability with at least one recognized peptide then those identifications were accepted. Using Protein Prophet algorithm (Nesvizhskii *et al.*, 2003) probabilities for the Protein pedtides were assigned. Similar peptides of the different SPATE proteins were selected and filtered out so as to obtain unique peptides for each SPATE and relative abundance was measured as a proportion of the normalized spectral counts obtained from the analysis as described in (Liebler, 2001; Lundgren *et al.*, 2010).

4.4.10 Statistcial Analyses

Experimental data were expressed as a mean ± standard error of the mean (SEM) in each group. For pairwise comparision, a two-tailed student t-test was used to analyze the means of group. For comparision of means of more than two population, analysis of variance (ANOVA) was used. For turkey infection experiments, the Mann–Whitney test was used to compare the samples by pairs, and the Kruskal–Wallis test was used to compare groups. A P value of <0.05 was considered statistically significant. All data were analyzed with Graph Pad Prism 7 software (GraphPad Software, San Diego, CA, USA).

Table 4.1 List of strains and plasmids used in this study.

S. N	Strains	Characteristic(s)	References
1	QT598	APEC O1: K1 (Sequence type, ST1385)	(Habouria *et al.*, 2019)
2	QT775	Δ*asd* λ *pir* *E. Coli* MGN-617	(Crépin *et al.*, 2012a)
3	QT4726	QT598 Δ*tagBC*::kan, Km^r	(Habouria *et al.*, 2019)
4	QT5182	Δ5 SPATEs or QT598 Δ*tagBC*Δ*vat*:: cat Δ*sha*:: kan Δtsh::tetAR(B) Cm^r Km^r Tc^r	(Habouria *et al.*, 2019)
5	QT5188	QT598Δ*tagBC*Δ*vat*::cat, Cm^r	(Habouria *et al.*, 2019)
6	QT5189	QT598Δ*tagBC*Δ*vat*::cat Δ*sha*::kan, Cm^r Km^r	(Habouria *et al.*, 2019)
7	QT5190	QT598Δ*sha*:: kan Δ*tsh*:: tetAR(B) Km^r Tc^r	(Habouria *et al.*, 2019)
8	QT5191	QT598Δ*tagBC* Δ*vat*:: cat Δ*tsh*:: tetAR(B) Cm^r Km^r Tc^r	(Habouria *et al.*, 2019)
9	QT5192	QT598Δ*sha*:: kan Km^r	(Habouria *et al.*, 2019)
10	QT5193	QT598Δ*tsh*:: tetAR(B) Tc^r	(Habouria *et al.*, 2019)
11	QT6023	pGP-tn7-Gm::*vat* transformed into λ *pir*-derivative *E. coli* Gm^r	This study
12	QT6024	pGP-tn7-Gm::*tagBC* transformed into λ *pir*-derivative *E. coli* Gm^r	This study
13	QT6025	pGP-tn7-Gm::*vat-tagBC* transformed into λ *pir*-derivative *E. coli* Gm^r	This study
14	QT6026	pGP-tn7-Gm::*sha* transformed into λ *pir*-derivative *E. coli* Gm^r	This study
15	QT6027	pGP-tn7-Gm::*tsh* transformed into λ *pir*-derivative *E. coli* Gm^r	This study
16	QT6028	pGP-tn7-Gm::*sha-tsh* transformed into λ *pir*-derivative *E. coli* Gm^r	This study
17	QT6030	pBCsk+::*sha-tsh* transformed into DH5α *E. coli* Km^r Gm^r	This study
18	QT6034	QT775+ pIJ615 Gm^r	This study
19	QT6035	QT775+ pIJ616 Gm^r	This study
20	QT6036	QT775+ pIJ617 Gm^r	This study
21	QT6037	QT775+ pIJ618 Gm^r	This study
22	QT6038	QT775+ pIJ619 Gm^r	This study
23	QT6039	QT775+ pIJ620 Gm^r	This study

24	QT6041	QT775+ pIJ623 Gm^r	This study
25	QT6042	QT5182 Δ5 SPATEs:: FRT Cm^r Tc^r	This study
26	QT6043	QT6042 + pIJ258 Cm^r Km^r Tc^r	This study
27	QT6044	QT6043*::vat* Cm^r Tc^r Gm^r	This study
28	QT6045	QT6043*::tagBC* Cm^r Tc^r Gm^r	This study
29	QT6046	QT6043*::vat-tagBC* Cm^r Tc^r Gm^r	This study
30	QT6047	QT6043*::tsh* Cm^r Tc^r Gm^r	This study
31	QT6048	QT6043*::sha* Cm^r Tc^r Gm^r	This study
32	QT6051	QT6043*::sha-tsh* Cm^r Tc^r Gm^r	This study
33	QT6052	QT6046*:: sha-tsh* Cm^r Tc^r Gm^r Km^r (5-way complemented strain)	This study
Plasmids			
1.	pIJ253	Cloning vector, pGp-Tn7-Gm	(Crépin *et al.*, 2012a)
2.	pCP20	FLP recombinase, Amp^r	(Datsenko *et al.*, 2000)
3.	pIJ258	Transposase expressing plasmid	(Crépin *et al.*, 2012a)
4.	pIJ266	Cloning vector, pBluescript II SK (+)	(Caza *et al.*, 2011)
5.	pMEG-375	Allelic exchange vector, pMEG-375	(Dozois *et al.*, 2003)
6.	pIJ615	pGP-tn7-Gm::*vat* Gm^r Amp^r	This study
7.	pIJ616	pGP-tn7-Gm::*tagBC* Gm^r Amp^r	This study
8.	pIJ617	pGP-tn7-Gm::*vat-tagBC* Gm^r Amp^r	This study
9.	pIJ618	pGP-tn7-Gm::*sha* Gm^r Amp^r	This study
10	pIJ619	pGP-tn7-Gm::*tsh* Gm^r Amp^r	This study
11.	pIJ620	pGP-tn7-Gm::*sha-tsh* Gm^r Amp^r	This study

12.	pIJ622	pBluescript II SK (+):: *sha_tsh* Gm^r Km^r	This study
13.	pIJ623	pMEG-375:: *lacZ'-sha-tsh-lacA'* Gm^r Km^r	This study

Table 4.2 List of primers used in this study.

S. N	Primer name	Direction	Characteristic(s)	Sequences 5'-3'
1	CMD2856	Vat-Forward	Gibson pairs to amplify *vat* gene with promoter from QT598 to clone it into *stuI* site of pGP-Tn7-gm. (Used with CMD2857)	TTGGGCCCGGTACCTCGCGAAGGCATAAGTCAAAACACCTGAG
2	CMD2857	Vat-Reverse	Gibson pairs to amplify *vat* gene with promoter from QT598 to clone it into *stuI* site of pGP-Tn7-gm. (Used with CMD2856)	TTCACTTATCTGGTTGGCCTGCAAGGCTCAACCTGAGTGTTACATG
3	CMD2860	Forward (TagBC)	Gibson pairs to amplify *tagbc* gene with promoter from QT598 to clone it into *XhoI* site of pGP-Tn7-gm. (Used with CMD2855)	GGCTGCAGGAATTCCTCGAGTGCTGCCATAGCTGAACCTG
4	CMD2855	Reverse (tagBC)	Gibson pairs to amplify *tagbc* gene with promoter from QT598 to clone it into *stuI* site of pGP-Tn7-gm. (Used with CMD2860)	TTCACTTATCTGGTTGGCCTGCAAGGGTAACATCACTACAGGCCCC

5	CMD2862	Forward (Vat-tagbc)	Gibson pairs to amplify *vat* gene with promoter from QT598 to clone it into *XhoI* site of pIJ616. (Used with CMD2881)	GGCTGCAGGAATTCCTCGAGCATAAGTCAAAACACCTGAG
6	CMD2881	Reverse Vat-tagbc)	Gibson pairs to amplify *vat* gene with promoter from QT598 to clone it into *XhoI* site of pIJ616. (Used with CMD2862)	AGGTTCAGCTATGGCAGCACTCGACTCAACCTGAGTGTTACATG
7	CMD2868	Forward (sha)	Gibson primer to amplify *sha* gene with promoter from QT598 to clone it into *stuI* site of pGP-Tn7-gm. (Used with CMD2869)	TTGGGCCCGGTACCTCGCGAAGGTCTGTTGAACTGGATTTGTGGG
8	CMD2869	Reverse (Sha)	Gibson primer to amplify *sha* gene with promoter from QT598 to clone it into *stuI* site of pGP-Tn7-gm. (Used with CMD2868)	TTCACTTATCTGGTTGGCCTGCAAGGGGTACCATTCAGAGTGACAGGATGGC
9	CMD2872	Forward (Tsh)	Gibson primer to amplify *tsh* gene with promoter from QT598 to clone it into *XhoI* site of pGP-Tn7-gm. (Used with CMD2875)	CCCGGGCTGCAGGAATTCCTCGAGTGTGCATAGCACACATTCGC
10	CMD2875	Reverse (Tsh)	Gibson primer to amplify *tsh* gene with promoter from QT598 to clone it into *stuI* site	TTCACTTATCTGGTTGGCCTGCAAGGATAGTCCCTTTGCTGCACAG

			of pGP-Tn7-gm. (Used with CMD2872)	
11	CMD2866	Forward (Sha)	Gibson primer to amplify *sha* gene with promoter from QT598 to clone it into *XhoI* site of pIJ619. (Used with CMD2867)	CCCGGGCTGCAGGAATTCCTCGAGTCTGTTGAACTGGATTTGTGGG
12	CMD2867	Reverse (Sha)	Gibson primer to amplify *sha* gene with promoter from QT598 to clone it into *XhoI* site of pIJ619. (Used with CMD2866)	TACCGGGCCCAAGCTTCTCGAATTCAGAGTGACAGGATGGC
13	CMD2964	Forward (GM_tn7for sk+)	Gibson primer to amplify *gm_sha_tsh* gene from pIJ620 to clone it into *KpnI* site of pIJ266. (Used with CMD2968)	TCACTATAGGGCGAATTGGGTACCTCCTGAGTAGGACAAATCCG
14	CMD2968	Reverse (Tsh_R2)	Gibson primer to amplify *gm_sha_tsh* gene from pIJ620 to clone it into *xhoI* site of pIJ266. (Used with CMD2964)	TCGATACCGTCGACCTCGAGATAGTCCCTTTGCTGCACAG
15	CMD2969	Forward (lacAgm)	Gibson primer to amplify *lacA'_km_gm_sha* gene from pIJ622 to clone it into *PacI* site of pMEG vector. (Used with CMD2889)	CGCGCCGGATCCTTAATTAGGGTGACGATACTACCCG

16	CMD2889	Reverse (shaRjoin	Gibson primer to amplify *lacA'_km_gm_sha* gene from pIJ622 to clone it into *PacI* site of pMEG vector. (Used with CMD2969)	TCGAATTCAGAGTGACAGGATGGC
17	CMD2888	Forward (tshFjoin	Gibson primer to amplify *tsh_lacZ'* gene from pIJ622 to clone it into *PmeI* site of pMEG vector. (Used with CMD28970)	ATCCTGTCACTCTGAATTCGAGTGTGCATAGCACAC
18	CMD2970	Reverse (LacR)	Gibson primer to amplify *tsh_lacZ'* gene from pIJ622 to clone it into *PmeI* site of pMEG vector. (Used with CMD2888)	TGCATGCCTGCAGGTTTGGTGCGCAGCCTGAATGGC
19	CMD1420	Forward	Screening primer of pIJ253 (pGP-Tn7-Gm)	TTCGGTCAAGGTTCTGGACCAGTT
20	CMD2897	Reverse	Screening primer for *vat*	TAAGGATTGTTGCCACCAACC
21	CMD2878	Reverse	Screening primer for *tsh*	ACACAGCAACAAGTTCACCTC
22	CMD2879	Reverse	Screening primer for *sha*	ACTTCCTGCAGAGAATAGTACC
23	CMD2880	Reverse	Screening primer for *tagbc*	TGGAATGTTGGTGGAACCTG

24	CMD96	Forward	Screening primer for confirmation of *vat* complementation.	AACGGTTGGTGGC AACAATCC
25	CMD97	Reverse	Screening primer for confirmation of *vat* complementation.	AGCCCTGTAGAAT GGCGAGTA
26	CMD2096	Forward	Screening primer for confirmation of *tagb* complementation.	GCTGCCATAGCTG AACCTGCG
27	CMD2097	Reverse	Screening primer for confirmation of *tagb* complementation.	CAGAGGCACGGCC ACTGAAC
28	CMD2100	Forward	Screening primer for confirmation of *tagc* complementation.	TGCAATGTGGGTAT GGAGTCG
29	CMD2101	Reverse	Screening primer for confirmation of *tagc* complementation.	GTGGCCTTCGCGT ATTTCC
30	CMD1785	Forward	Screening primer for confirmation of *sha* complementation.	GCCTGAACATCGG CTTCAAGA
31	CMD1786	Reverse	Screening primer for confirmation of *sha* complementation.	TCAGAACTCATATC GAATACCGAC
32	CMD100	Forward	Screening primer for confirmation of *tsh* complementation.	AGTCAGGGGGATG CACAGAAA

33	CMD101	Reverse	Screening primer for confirmation of *tsh* complementation.	GCGGTTCTCCCAG TCCTCC
34	CMD2188	Forward	*sha* qPCR primer	GATAGTGGTTCTCCG CTCTTTG
35	CMD2189	Reverse	*sha* qPCR primer	AGCCCAGTTGTTTGC TCTAC
36	CMD2328	Forward	*tagB* qPCR primer	GGGAGACGGAACCG TTATATTG
37	CMD2329	Reverse	*tagB* qPCR primer	CGTCGTTTAGCACCA GAGTAG
38	CMD2330	Forward	*tagC* qPCR primer	GCCGGGTTCAGATTG GAAAG
39	CMD2331	Reverse	*tagC* qPCR primer	GTACGGGCATCCAG GTATTATC
40	CMD2332	Forward	*vat* qPCR primer	AGCACGAACTGGGAA GTATG
41	CMD2333	Reverse	*vat* qPCR primer	ACAGACCACTGGCAT AGAAAC
42	CMD2334	Forward	*tsh* qPCR primer	ACTCCACGGCAGGAA ATATG
43	CMD2335	Reverse	*tsh* qPCR primer	TGAACCGCGTCCAGA TTATC

4.5 Results

4.5.1 Single-copy complementation of SPATE-encoding genes in the ∆5SPATEs mutant of APEC QT598.

To ascertain the potential role of each of the 5 different SPATEs during APEC infection in the turkey, different individual SPATE-encoding genes as well as re-introduction of all 5 SPATE-encoding genes was undertaken to determine if the Δ5SPATEs mutant could regain the infective capacity of the wild-type parent strain QT598. To achieve this, we used the Tn7 transposon system, to re-introduce SPATE encoding genes in single copy by transposition at the conserved *att*Tn7 site in chromosome of Δ5SPATEs strain (QT6042). The SPATE-encoding genes *vat, tagBC, tsh, sha,* and also *vat+tagBC or sha+tsh* were cloned into the pGP-miniTn7-Gm vector (**Figure 4.1A)**. The constructs were then transformed into *E. coli* strain MGN-617 as a donor strain for conjugation into the Δ5SPATEs mutant containing the Tn7 transposase-encoding plasmid pIJ258, as a recipient strain. Conjugation was performed using the approach described by (Crépin *et al.*, 2012a). Following conjugation, the transposition at the *att*Tn7 site was mediated by the *tnsABCD* encoded transposase system, where TnsAB excise and direct the Tn7 segment containing the SPATEs genes and TnsCD promote the integration of the segment into the *attTn7* site of recipient strain (**Figure 4.1B**). Confirmation of integration of SPATE-encoding genes at the *att*Tn7 site was verified by PCR using the primer pairs listed in **table 4.2**.

To generate a fully complemented strain of the Δ5SPATEs mutant, we used an allelic exchange method to re-introduce both the *sha* and *tsh* genes into the *lac* genes on the chromosome of strain QT6046, which is the Δ5SPATEs strain containing the *tagBC* and *vat* genes integrated within the *att*Tn7 site. Allelic exchange into the *lac* genes was achieved using the *sacB*-based counterselection with a suicide plasmid as described in (Porcheron *et al.*, 2014). Integration of SPATE genes at the *lac* genes were verified by PCR using primer pairs (CMD1420/CMD2878), and production of white colonies on MacConkey agar plates confirming disruption of the *lac* genes. The fully complemented Δ5SPATEs mutant strain therefore contained single copies of the *vat and tagBC genes* at the *attTn7* site and of the *sha* and *tsh genes* within the *lac* region resulting in a 5-way SPATEs complemented strain (QT6052).

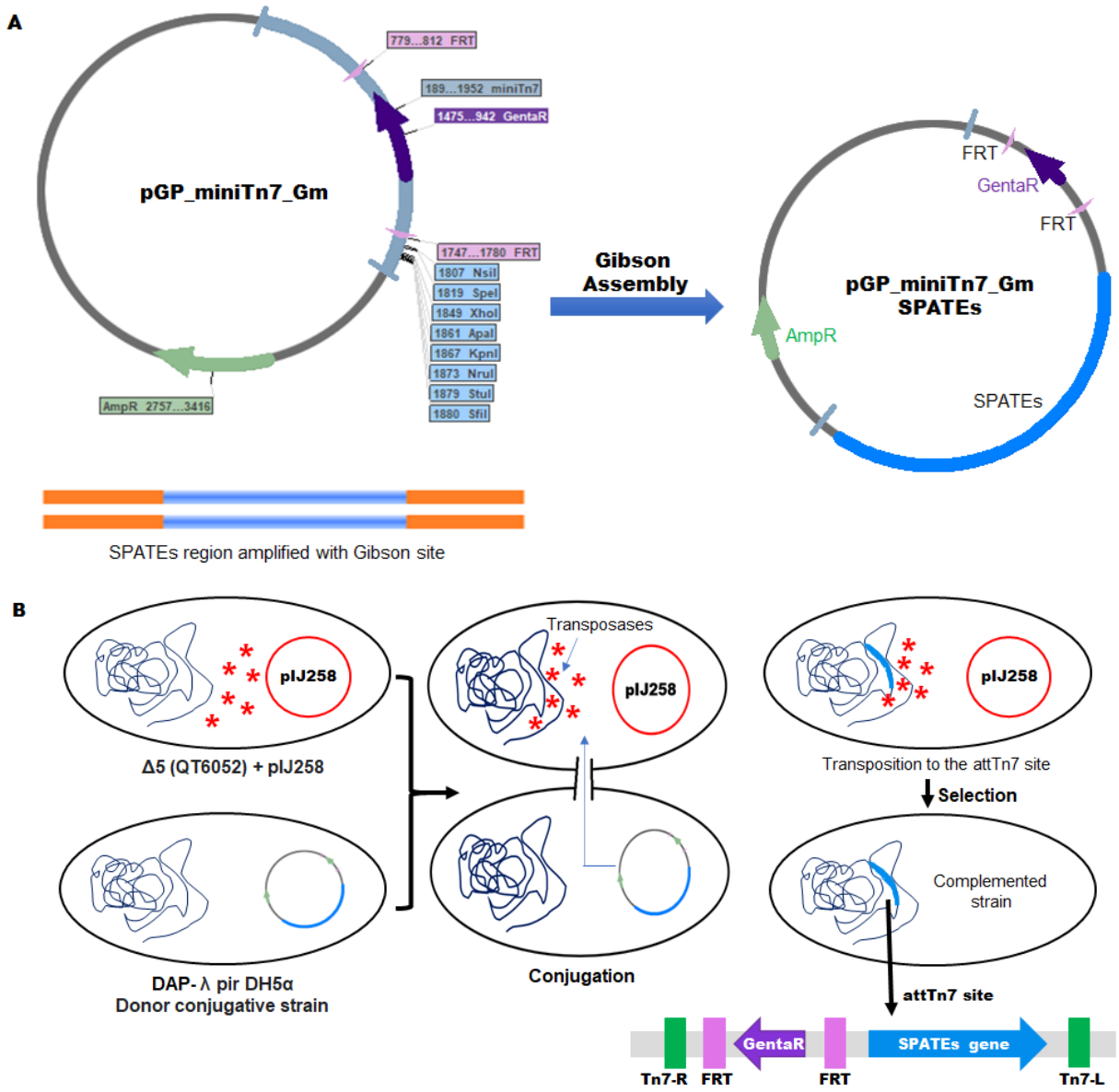

Figure 4.1 Illustration displaying the complementation strategy.

A) Schematic representation of cloning vector pGP-mini-Tn7-Gm, a mobilizable suicide vector, where the multiple cloning site (MCS) is located between the two Tn7 ends and *XhoI* and *StuI* sites were used for cloning of SPATEs genes. This construction allowed Tn7 mediated transposition of SPATEs genes. B) Schematics for *attTn7* site specific integration of SPATEs genes. The Δ*asd* donor strain MGN-617 (which requires DAP for growth) was conjugated with recipient strain containing plasmid pIJ258 that expresses the TnsABCD transposases which in turn drive the integration of genes into the *attTn7* site.

4.5.2 Expression and production of cloned SPATEs in culture supernatants.

To complement the Δ5SPATEs mutant, it was important to verify secretion and bioactivity of each of the cloned SPATE genes that were used to introduce these genes in single copy to the *att*Tn7 site. To verify production of the SPATEs that were cloned into the pGP_miniTn7_Gm vector, each of the clones were tested in *E. coli* strain DH5α λpir for the ability to produce SPATEs in culture supernatants. Vectors encoding different SPATE-encoding genes *(vat, tagBC, sha, tsh, vat+tagBC, sha+tsh)* expressed from their native promoter regions, were all shown to produce high-molecular weight proteins >100 kDa that corresponded to the size of the secreted SPATE proteins and a clone containing the empty vector (pGP_miniTn7_Gm) was included as negative control. (**Figure 4.2A**). Importantly, in strain DH5α *λpir* the cloning vector is able to replicate, whereas it is not in any of the APEC strain derivatives. Further, protein samples from supernatants of APEC wild-type parent strain QT598 and the Δ5SPATEs isogenic mutant (QT6042) were included as additional positive and negative controls.

The Coomassie SDS-PAGE analysis clearly demonstrated the presence of proteins corresponding to the size of the SPATEs in strain QT598 and in each of the *E. coli* DH5α λ-*pir* clones containing SPATE-encoding genes. By contrast, the supernatants of *E. coli* DH5α λ-*pir* containing the empty vector and Δ5SPATEs mutant did not contain any secreted proteins corresponding to the SPATEs (**Figure 4.2A**). These results confirmed that each of the cloned copies of the different SPATEs introduced into the pGP_miniTn7_Gm plasmid encoded functional SPATEs that were effectively secreted into the culture medium.

4.5.3 Complemented strains of the ∆5SPATEs mutant all produced and secreted SPATEs *in-vitro*.

Since we confirmed that each of the cloned copies of the different SPATEs produced secreted proteins in DH5α λ-*pir*, these vectors were used to introduce these genes into the Δ5SPATEs mutant. For each of the different complemented strains, we determined if SPATEs proteins were present in supernatants by Western blot analysis. As a primary antibody, we used the polyclonal serum raised in rabbit against the passenger domain of the Vat autotransporter. This serum is known to cross-react with all five SPATEs (Pokharel *et al.*, 2020), and we identified SPATEs in the culture supernatants of each of the complemented strains (**Figure 4.2B**). We clearly observed the expression of SPATEs in the QT598 wildtype as positive control and absence of SPATEs in the Δ5 SPATEs mutant (QT6042). In addition, all five SPATE complemented mutant strains (*sha*, *tsh*, *sha+tsh*, *vat*, *vat+tagBC* and *tagBC*) showed presence of SPATEs in each of the culture

supernatants (**Figure 4.2B**). These results confirmed complementation of the production of each of the SPATEs when introduced in single copy into the genome of the Δ5 SPATEs mutant strain.

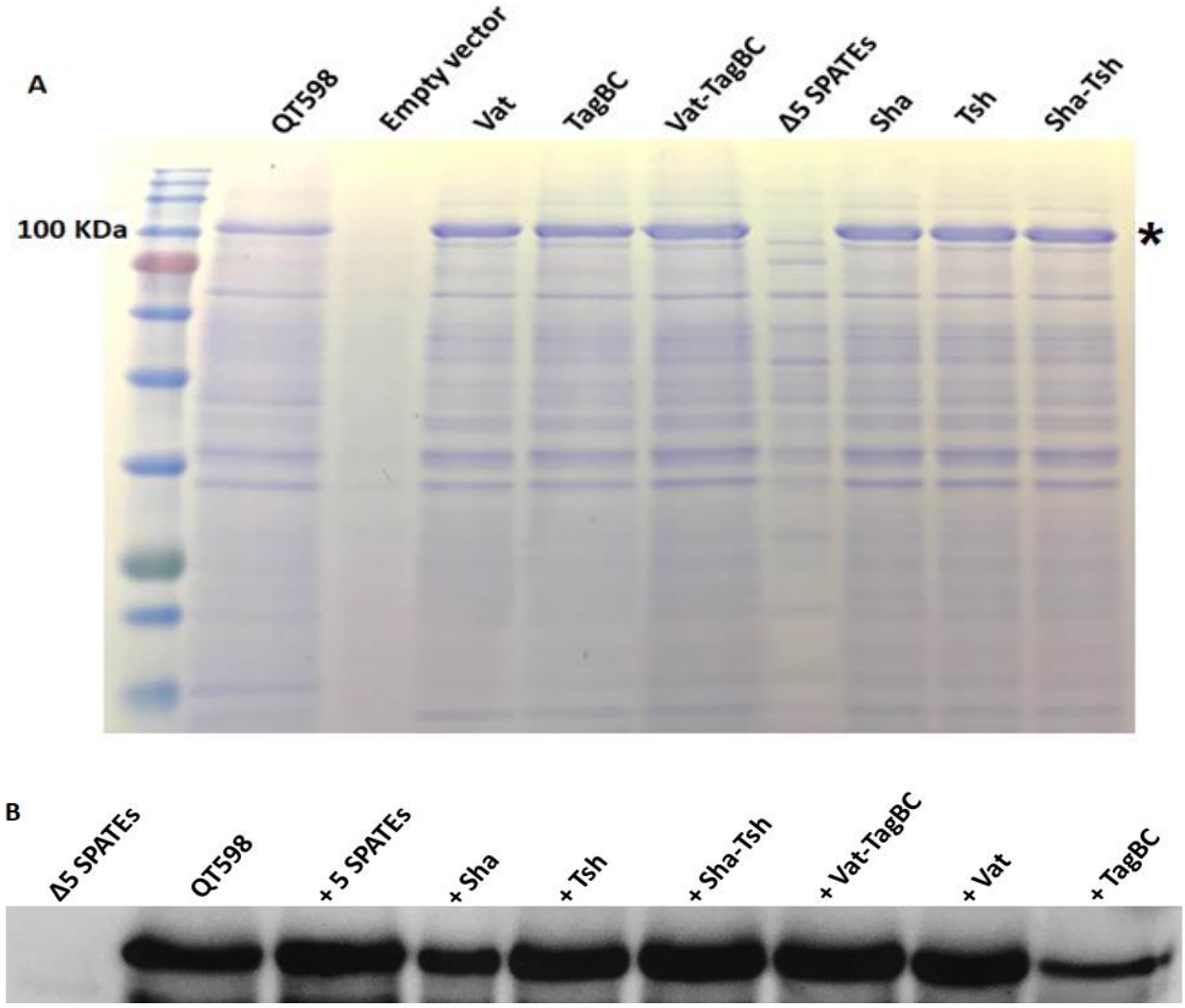

Figure 4.2 Visualization of SPATE proteins from clones by SDS-PAGE and verification of SPATE proteins in complemented strains using western blot.

A) DH5α λ-*pir* with medium-copy plasmid pGP_miniTn7_Gm_SPATEs clones expressing SPATE proteins were migrated alongside protein extracted from QT598 (wildtype), QT6042 (Δ5SPATEs), empty vector (pGP_miniTn7_Gm) and protein marker (17-245 kDa) and stained with Coomassie blue. SPATEs proteins can be observed at slightly above 100 kDa. B) Autotransporter proteins expressed in SPATEs complemented strains, QT598 wildtype and QT6042 (Δ5SPATEs) were visualized in western blot using a SPATEs conserved region specific polyclonal serum as primary antibody and HRP-anti-rabbit IgG(H+L) as secondary antibody (Novus).

4.5.4 Autoaggregation ability is retained in the clones.

In addition to confirmation of presence of SPATEs proteins in the supernatants of the complemented strains by SDS-PAGE, Western blot and mass spectrometry; we also investigated some phenotypes associated with specific SPATEs proteins. It was previously shown that TagB, TagC and Sha proteins when cloned in *E. coli* K-12 can mediate autoaggregation (Habouria *et al.*, 2019). Autoaggregation tests using the DH5α λ-*pir* SPATE-encoding clones demonstrated that clones containing pGP-Tn7-Gm *tagBC* or *sha* produced autoaggregation, whereas this was absent in clones encoding either *tsh* or *vat* (**Figure 4.3**). Thus, as had been observed previously the autoaggregative phenotype of the *sha* and *tagBC* encoding clones was retained, whereas this phenotype was absent in clones expressing either Vat or Tsh proteins (Habouria *et al.*, 2019). Despite, the capacity of either *sha* or *tagBC* to increase aggregation in the *E. coli* K-12 cloned strain background, complementation of the Δ5 SPATEs mutant with these genes did not have any appreciable effect on autoaggregation and this phenotype was also absent from the APEC wild-type strain QT598 (**Figure 4.3**). As such, the cloned copies of either *sha* or *tagBC* cloned into pGP_miniTn7_Gm retained the distinct autoaggregation phenotype previously observed, and this phenotype was not observed in clones encoding either Tsh or Vat proteins.

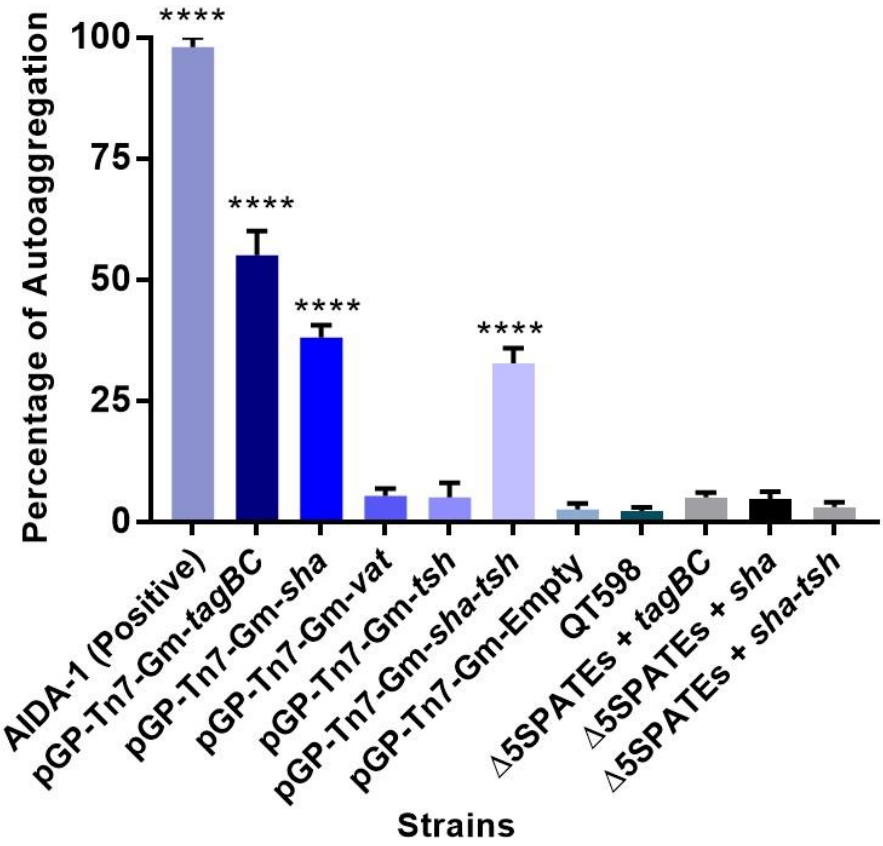

Figure 4.3 Autoaggregation phenotypes of clones

Individual SPATE genes were cloned into pGP-Tn7-Gm vector for the complementation purpose. *E. coli* DH5 alpha lambda pir with these individual vectors expressing different SPATEs were grown overnight and adjusted to OD_{600} of 1.5 and left to sediment at 4°C. Samples were taken at 1 cm from the top surface of the cultures after 3 h to determine the change in OD_{600}. Assays were performed in triplicate, and the rate of autoaggregation was assessed by a decrease in the OD_{600} indicating an increase in bacterial sediments that settle at the bottom of culture tubes. Empty pGP-Tn7-Gm vector was used as a negative control and the AIDA-1 autotransporter as a positive control for autoaggregation. Error bars represent standard errors of the means and p value is generated by comparing to empty pGP-Tn7-Gm using one-way ANOVA.

4.5.5 Complemented SPATEs have active serine protease sites.

SPATE proteins contain serine protease catalytic domains which are an important attribute of their bioactivity. To confirm that the cloned SPATEs were catalytically active, we conducted enzymatic assays for all of SPATE complemented derivatives of Δ5SPATEs mutant using a substrate cleavage specificity assay. Different members of SPATE family are known to have proteolytic activity on synthetic peptides (Benjelloun-Touimi *et al.*, 1998; Dutta *et al.*, 2002). The high-molecular-weight supernatant fractions of each SPATE complemented strain of Δ5SPATE

mutant were incubated with N-Succinyl-Ala-Ala-Ala-p-nitroanilide (elastase substrate), N-Benzoyl-L-arginine 4-nitroanilide (trypsin substrate) or N-succinyl-ala-ala-pro-phe-p-nitroanilide (chymotrypsin substrate) (Sigma-Aldrich, St. Louis, MO, USA). Trypsin-like activity was demonstrated in the Δ5SPATE mutant complemented with either *tagB*, *tagC* or both *tagB* and *tagC* genes, as supernatant extracts from these strains efficiently cleaved N-Benzoyl-L-arginine 4-nitroanilide (**Figure 4.4**). In addition, the Δ5SPATE mutant complemented with either *sha*, *vat* or *tsh* genes demonstrated significant elastase-like activity toward N-Succinyl-Ala-Ala-Ala-p-nitroanilide (**Figure 4.4**). The extracts of supernatant fractions from wild-type strain QT598 showed both trypsin-like and elastase-like activities whereas supernatant extracts from the Δ5SPATEs mutant did not show any appreciable level of protease activity on these substrates. Taken together, these results confirm that each of the cloned SPATEs when introduced in single copy into the genome of Δ5SPATEs mutant demonstrated functional protease cleavage activity, whereas this type of protease activity was greatly reduced in the Δ5SPATEs mutant.

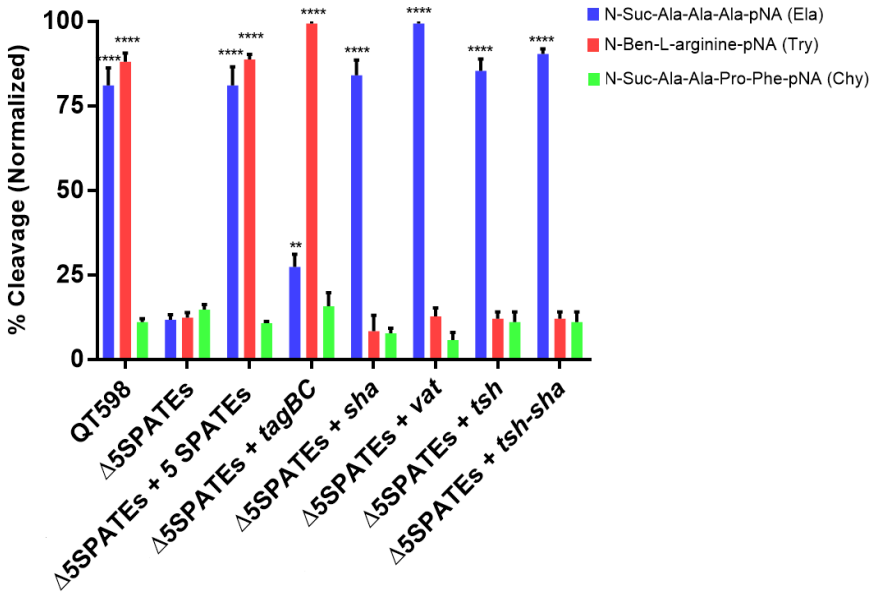

Figure 4.4 Oligopeptide cleavage activity of complemented SPATEs.

Five µg per ml of each SPATE-containing supernatant from different SPATE complemented QT598 ΔSPATE strains were incubated at 37°C for 3 h with 1mM of synthetic chromogenic oligopeptide substrates specifically recognized by the following enzymatic activities: Elastase (Ela)-(N-Suc-Ala-Ala-Ala-pNA); Trypsin (Try)-(N-Ben-L-arginine-pNA); or Chymotrypsin (Chy)-(N-Suc-Ala-Ala-Pro-Phe-pNA). Activity was measured via released para-nitroaniline and normalized to the maximum absorbance value. Data are the means of three independent experiments, and error bars represent the standard errors of the means (Non-specific activity (i.e., relative activity <10 %), *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001 Two-way ANOVA with multiple comparisons vs QT598 Δ5 SPATEs mutant).

4.5.6 Investigating the role of different SPATEs in an APEC experimental infection model in turkeys

APEC can infect numerous avian species including chickens, turkeys, and ducks. Among infection models that have been used, the chicken is the most common model, although both ducks and turkeys have been used in some reports (Antao *et al.*, 2008; Sadeyen *et al.*, 2014; Wang *et al.*, 2022c). Since the source of strain QT598 was originally from a 4-day-old turkey poult, it was of interest to investigate whether the turkey could provide a valid natural host infection model for APEC strain QT598. Further, since there are 5 distinct SPATEs in this strain and some of these SPATE encoding genes such as *tagBC* were mainly identified in APEC isolated from turkeys (Habouria *et al.*, 2019), we speculated that the turkey infection model could also provide some insight into the potential roles of specific SPATEs during the infectious process that may be more host-specific to the turkey.

To determine if strain QT598 was able to infect systemic organs following air-sac inoculation in turkeys, we adapted a model we have used frequently in 3-week-old chickens (Dozois *et al.*, 2000) for use in a 6-day-old turkey poult model. In the air-sac infection model, wild-type strain QT598 was shown to be present in high numbers in the lungs of infected poults (from 10^6 to 10^7 CFU/g with a median of 4×10^6) and was also isolated from most samples taken from the spleen and liver (median of 1×10^3 and 2×10^3 respectively in these tissues) at 48 hours post-infection **(Figure 4.5A-C).** We also tested for presence of bacteria in the blood 6 h and 48 h after infection, however all samples tested were negative, indicating that strain QT598 can infect the lungs at high levels and infect spleen and liver, but is unable to proliferate or effectively persist in the blood of turkeys. Remarkably, despite the high levels of bacterial numbers in the lungs, the poults showed no, or few symptoms associated with colibacillosis, with no evident signs of respiratory stress, huddling, or lethargy. The only evident sign of infection identified was a reduced level of weight gain that was observed in the poults infected with QT598 when compared with turkeys infected with mutants lacking all or some of the SPATE-encoding genes (**Figure 4.5D**).

Using this model to investigate the role of all 5 SPATEs in the respiratory infection model of turkeys, we first evaluated the potential of the Δ5SPATEs mutant (QT6052). The mutant strain lacking all SPATE genes had significantly less bacterial numbers in both the lungs and liver (**Figure 4.5A and C**) when compared to the wild-type parental strain QT598, with a median of 1000-fold less in the lungs and 10-fold less in the liver. The Δ5SPATEs mutant also showed a decreased median number in the spleen, although due to variation in bacterial numbers in the samples this difference between groups was just above the significance threshold (P=0.06) (**Figure 4.5B**). The % weight

gain of the poults infected with the Δ5SPATEs mutant was also significantly higher when compared to the % weight gain of the poults infected with parent strain QT598 (**Figure 4.5D**).

As several different genetic manipulations and bacterial passages were required to generate the Δ5SPATEs mutant, it was important to ascertain if re-introduction of the SPATE-encoding genes could restore infective capacity. We therefore generated a strain wherein all five of the distinct SPATEs were re-introduced into the Δ5SPATEs mutant. This 5-way complemented strain (QT 6048) effectively regained the capacity to colonize the lung and liver in higher numbers at levels comparable to poults infected with parent strain QT598 (**Figure 4.5A and 4.5C**). Further, the median weight gain of poults infected with this strain was also significantly reduced compared to the Δ5SPATEs mutant (P=0.008) (**Figure 4.5D).** Taken together, these results demonstrate that re-introduction of the 5 SPATE-encoding genes resulted in complementation of the infective capacity of strain QT598, fulfilling Koch's molecular postulates and demonstrating an important cumulative role for SPATEs in infection of turkey poults by APEC strain QT598.

4.5.7 Contribution of specific SPATEs for APEC infection of turkey poults

Since it was possible to generate strains of the Δ5SPATEs mutant that were only complemented with one or a subset of some of the SPATE-encoding genes, this provided an opportunity to investigate if re-introduction of only a certain subset of SPATE-encoding genes could restore infective capacity to the SPATE-negative mutant. Complementation with only the individual *sha*, *vat,* or *tsh* genes or the *tagBC* genes did not result in any significant regain of bacterial numbers in the lungs of infected poults compared to the Δ5SPATEs mutant (**Figure 4.5A**). However, re-introduction of both of the ColV-plasmid encoded SPATEs *tsh* and *sha* resulted in a significant gain in numbers in the lungs at a similar level to poults infected with wild-type strain QT598 (**Figure 4.5A**).

Interestingly, introduction of either the *tagBC* genes or the *vat* gene also resulted in a significant regain of bacterial numbers in the liver to levels similar to poults infected with QT598 (**Figure 4.5C**). No significant differences in colonization of infected strains in spleen were identified, as nearly all strains had similar bacterial numbers in these tissues. The turkeys were weighed to determine the percentage of gain of each of the poults during the course of the infection study, Notably, the Δ5SPATEs mutant demonstrated a clear level of weight gain compared to the wild- type strain QT598 ($p = 0.001$). Compared to the group infected with Δ5SPATEs mutant complementation with several different SPATE-encoding genes alone or in combination resulted in a significant decrease in percentage weight gain of animals including the 5-way complemented strain ($p = 0.008$), or the SPATE complemented only with the *sha* gene ($p = 0.013$), only with the *tsh* gene ($p = 0.001$)

or with *sha* and *tsh* genes (p = 0.001) complemented strains. However, the median percentage weight gains of poults infected with the SPATE mutant complemented with either *vat* or *tagBC* genes were not significantly lower.

Taken together, infection experiments using the SPATE-negative mutant complemented with a variety of different SPATE genes, has demonstrated a role for *sha* and *tsh* combined for infection of the lungs, and a predominant role for *tsh* and *sha* individually or in combination for decreased weight gain of infected birds. Further, a role for *vat* and *tagBC* for increased infection of the liver was demonstrated using the complemented mutant infection model. However, none of the Δ5SPATEs mutant derivatives that were complemented with only partial subsets of SPATE encoding genes demonstrated a regain that was as marked as the strain completed with all 5 SPATEs.

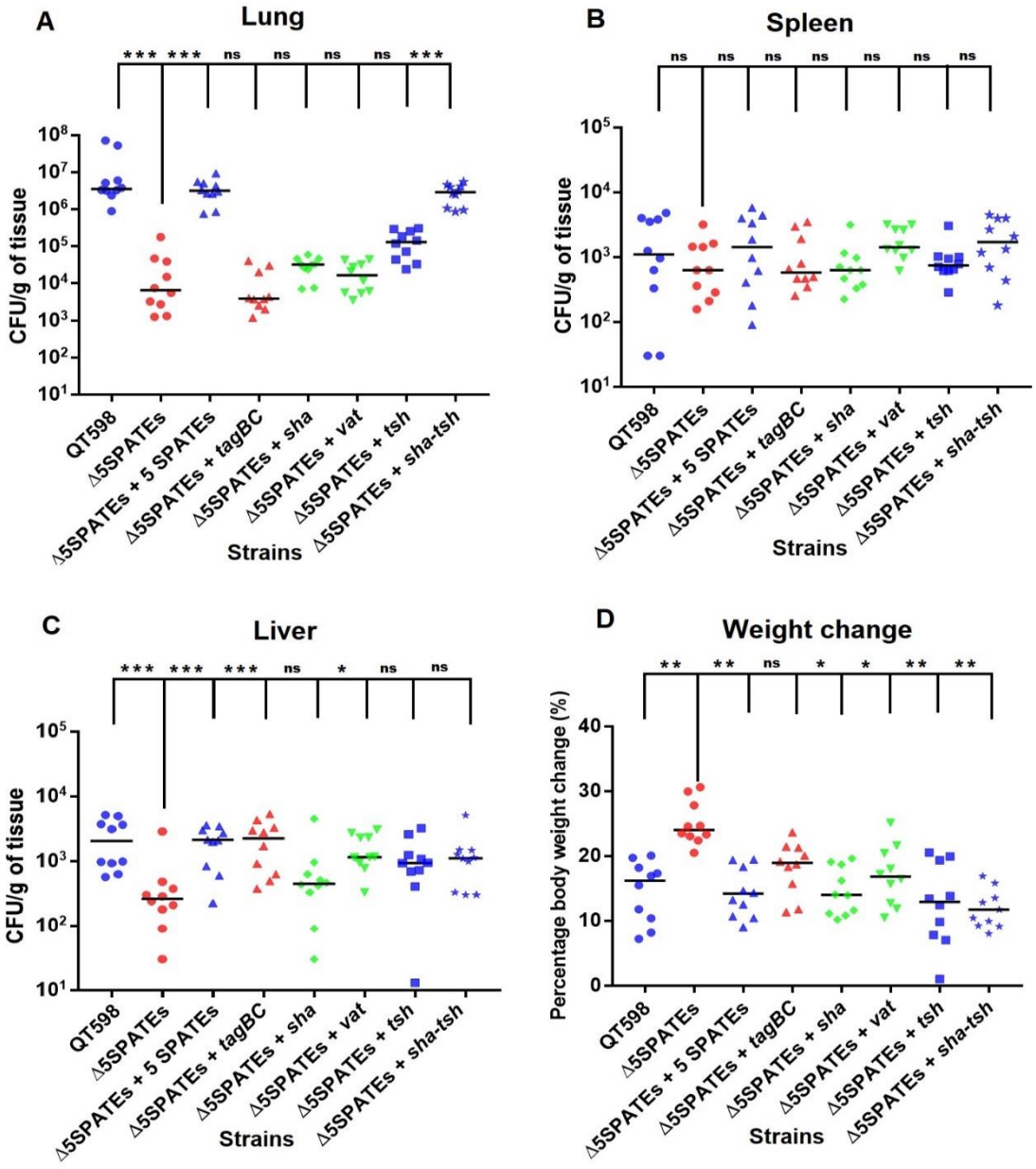

Figure 4.5 Role of SPATE encoding genes in a turkey model of airsac infection.

6-day old turkeys were challenged via airsac infection with wild-type QT598, Δ5SPATEs mutant (wherein all 5 SPATE genes are inactivated) and different SPATE-encoding gene complemented derivatives of the Δ5SPATEs mutant (A-D). Turkeys were euthanized after 48 h post-infection, and lungs, liver and spleen were harvested for colony counts. Bacterial numbers present in the lungs (A), spleens (B), and livers (C) of turkeys infected are reported as CFU per gram of tissue. Data points represent bacterial counts from tissues isolated from different turkeys (n=10) 48h. P value was calculated using Kruskal Wallis Test against QT598 Δ5SPATEs. (ns p > 0.05, *p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001).

4.5.8 Expression of the *sha* and *tsh* genes are increased in turkey respiratory tissues.

Since we observed a potential role for some of the SPATE-encoding genes for infection in the turkey model, we wished to investigate if there were differences in expression of these SPATE-encoding genes by strain QT598 when grown under different culture conditions or during infection of the turkey respiratory tract. As bacterial numbers in the lungs were elevated following infection with QT598, it was possible to investigate expression of the different SPATEs *in vivo* in both the air sacs and the lung tissues **(Figure 4.6**). The levels of expression of different SPATEs were compared to growth in LB to an O.D.$_{600}$ of 0.6. When grown on minimal glycerol medium the expression of *vat* gene increased considerably (by 12-fold), whereas expression levels of other SPATE-encoding genes remained similar or decreased (**Figure 4.6**). Interestingly, in turkey respiratory tissues both the *sha* and *tsh* genes demonstrated a marked increase in expression compared to in vitro culture in LB, with *sha* demonstrating a 9-fold increase in air sacs and 11-fold increase in the lungs. Similarly, for *tsh* the level of expression was increased by 15-fold in the air sacs and increased by 27-fold in the lungs. Taken together, the increased expression of the *tsh* and *sha* genes of QT598 in infected respiratory tissues also supports a predominant role for these two autotransporters in APEC respiratory infection of turkeys.

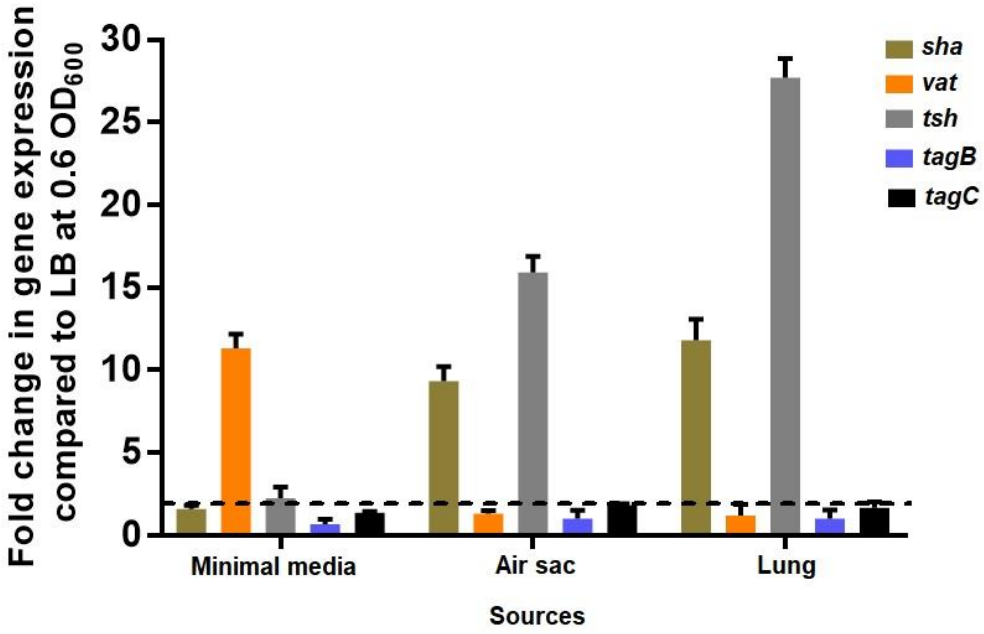

Figure 4.6 qRT-PCR analysis of SPATEs expression *in vivo* and *in vitro*.

Growth in rich medium (LB) to OD$_{600}$ of 0.6 was used as a standard and compared to growth in M9 glycerol minimal medium (supplemented with glycerol) at 37 °C and from air sac and lungs' tissue samples from infected turkeys.

The dashed line corresponds to the cut-off for a significant difference in expression. Error bars represent standard deviation of the mean, data shown are means ± SD (n = 4).

4.5.9 Proteomic analysis of culture supernatants of APEC QT598 and various SPATE mutants.

As APEC strain QT598 produces a quintet of SPATE proteins that are secreted into the culture medium during growth, we wished to investigate what the proportional levels of SPATE proteins were in both the wild-type strain and in different types of SPATE mutants (**Figure 4.7**). Proteomics analysis established that for wild-type strain QT598 grown in minimal M9 glycerol medium, Tsh was the predominant SPATE representing close to 60% of the total SPATEs, Vat was the second most abundant comprising close to 20% of the SPATEs, Sha represented 10% of the SPATEs and TagB and TagC contributed to around 1.5% and 7.0% respectively of total secreted SPATEs.

The deletion of *tsh* from QT598 resulted in an important increase in the relative abundance of Sha and Vat proteins in the supernatant as well as an increased proportion of TagB protein, with a lower proportion of TagC protein in the sample. Deletion of *sha* did not have any appreciable effects of relative levels of Tsh and Vat proteins, but TagB levels increased considerably compared to levels seen in supernatant of QT598. Deletion of *tagBC* resulted in a slight increase in proportion of Tsh with a decreased proportion of Sha protein. Finally, deletion of *tsh* and *sha*, resulted in a major increase in the proportion of Vat to over 75% of total SPATE composition, and a greater level of both TagC and TagB proteins compared to wild-type proportions.

Taken together these results indicate that Tsh, Sha, and Vat are the predominant SPATEs produced under these culture conditions. Further, the proteomic analyses demonstrate some differential levels of relative abundance of certain SPATEs in distinct deletion mutants, suggesting that there may be a degree of regulatory crosstalk or interactive roles of SPATE proteins among each other that can alter levels of production and secretion of specific SPATEs due to the deletion or loss of other SPATE-encoding genes.

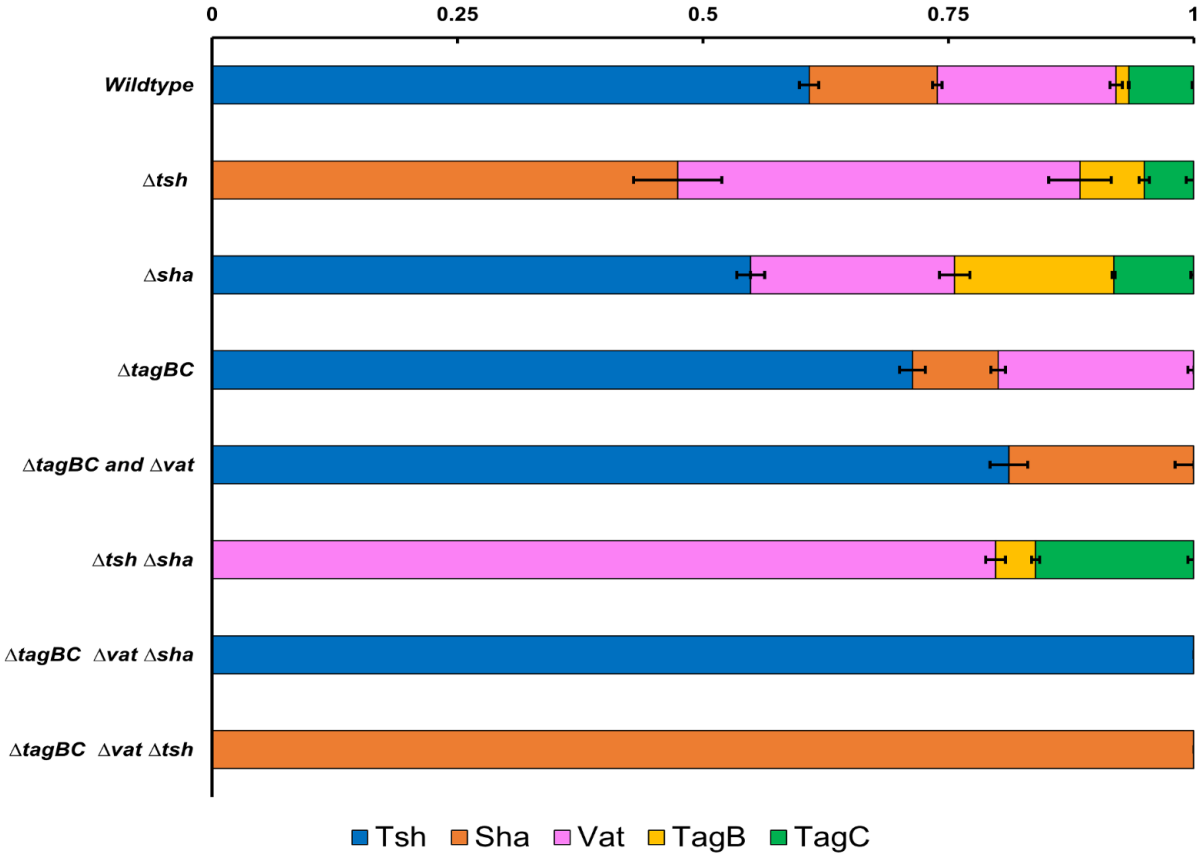

Figure 4.7 Relative abundance of SPATE proteins secreted by wild-type APEC QT598 and different SPATE deletion mutant derivatives.

Relative abundance of SPATEs secreted by QT598 strain and different mutants grown at 41°C, avian body temperature, overnight in minimal M9 glycerol medium to OD_{600} of 0.9-0.95. Extracted proteins were analyzed using MS-MS and the data were processed using scaffold software. Error bars represent standard error of the means, data shown are means of normalized unique spectral counts ± SEM (n = 3 for wildtype; n=2 for mutants).

4.6 Discussion of manuscript

SPATEs have diverse virulence functions, and can contribute to survival and virulence of pathogens such as *E. coli* in humans and other animal hosts (Pokharel *et al.*, 2019). In the current report, we have investigated the potential role of multiple SPATEs produced by an avian pathogenic *E. coli* strain QT598 in a turkey air sac infection model.

Following the identification of five distinct SPATEs from strain QT598 and demonstration of a competitive advantage of these SPATEs in the kidney in a murine urinary tract infection model (Habouria *et al.*, 2019), we were interested to study the role of these SPATEs in avian (chicken) air sac infection model (Dozois *et al.*, 2003). Although preliminary tests in the chicken indicated that strain QT598 was not effective at systemic infection in the three-week-old chicken model. This could possibly due to genomic differences compared to other APEC or ExPEC strains that may be able to cause disease in multiple host species and that some APEC strains may have a more host-species specific tropism (Zhang *et al.*, 2019). This may also be due to functional differences between the chicken and turkey immune defenses and physiology (Arsenault *et al.*, 2014). In addition, when considering the distribution of some of the SPATEs genes identified in strain QT598, we determined that both the *tagB* and *tagC* SPATE-encoding genes were only present in certain strains isolated from turkeys, but were absent from strains isolated from either chickens or ducks (Habouria *et al.*, 2019). This also suggested certain APEC strains may be more adapted or able to infect the turkey host. Since APEC QT598 was initially isolated from a young turkey poult, herein we investigated the potential role infective capacity of this APEC strain in a turkey of young age, using an air sac inoculation model in 6-day-old poults.

In this work, we demonstrated that loss of all five SPATEs from strain QT598 resulted in a significantly reduced bacterial burden in the lungs and liver. Further, the re-introduction of all 5 SPATE-encoding genes complemented the infective capacity of the SPATE-negative mutant, fulfilling molecular Koch's postulates regarding a collective role for these genes for systemic infection in the turkey model. In addition, we also showed a functional hierarchy or potential advantage of specific SPATEs for different host organs. Notably, *tsh* and *sha* together were found to contribute to increased infection of the lungs. In addition, the re-introduction of these genes alone or together into a SPATE-negative mutant also significantly reduced weight gain in poults. Interestingly, introduction of either the *vat* gene or *tagBC* genes to a SPATE-negative mutant, although they did not significantly increase bacterial numbers in the lungs, they did significantly increase bacterial burden in the liver. Taken together, these results support a collective role for SPATEs produced by strain QT598 with a predominant role for *tsh* and *sha* for infection of the lungs.

The *tsh* and *sha* genes are encoded on a ColV plasmid (pEC598-01) present in strain QT598 and both of these proteins belong to class 2 family of SPATEs (Habouria *et al.*, 2019). Numerous ColV-plasmids genes like *iroN*, *ompT*, *hlyF*, *iss, iutA* are regarded as predictors of APEC virulence in poultry (Johnson *et al.*, 2006), and demonstration of an important role of the Col-V plasmid encoded SPATEs further indicates how such transmissible plasmids can contribute to virulence of ExPEC in infections in both poultry and mammals including humans (Ewers *et al.*, 2007; Johnson *et al.*, 2008a; Johnson *et al.*, 2008b; Skyberg *et al.*, 2008; Waters *et al.*, 1991).

Tsh was the first SPATE protein identified in pathogenic *E. coli* and was originally characterized from an APEC O78:K80 strain χ7122 (belonging to sequence type ST23) that was derived from a clinical isolate originally isolated from a turkey (Dziva *et al.*, 2013; Provence *et al.*, 1994). Interestingly, a SPATE nearly identical to Tsh, described as Hbp (which differs at only 2 amino acids), was also identified in a ColV-plasmid from human ExPEC strain EB1 that was isolated from a wound abscess. Hbp was shown to contribute to virulence and in a co-infection abscess model with *Bacteroides fragilis* (Otto *et al.*, 2005; Otto *et al.*, 2002; Ruiz-Perez *et al.*, 2014), indicating that Tsh/Hbp can play a role in infections in both mammals and poultry.

The potential role of *tsh* for APEC infection has been called into question in some reports, since this SPATE gene was only shown to be present in 50% of APEC strain and is absent from the ColV plasmid of some virulent APEC strains. However, initially, deletion of *tsh* from strain χ7122 was shown to reduce the severity of the lesions in the air sacs of chickens (Dozois *et al.*, 2000). Since other APEC strains often encode additional SPATEs, such as Vat, it is not surprising that Tsh may not be present in some APEC strains. In another APEC O78 strain, Ec222, (Parreira *et al.*, 2003) reported that deletion of the *vat* gene resulted in attenuation in either a respiratory infection model or a cellulitis infection model. The *tsh* gene is absent from strain Ec222 (Personal communication, C. L. Gyles), whereas the *vat* gene is absent from APEC strain χ7122 (Restieri *et al.*, 2007).

This is the first indication of a contribution of the *sha* gene for infection in poultry, here in combination with *tsh* for infection of lungs in the turkey. The Sha protein is encoded on a distinct region of pEC598 adjacent to genes encoding a P-like (PL) fimbrial adhesin. Although the Sha protein has 43% identity/56% similarity with Tsh, it is likely they exhibit some shared and some distinct activities. Both Sha and Tsh were shown to act as mucinases (Pokharel *et al.*, 2020), but only Sha exhibited bacterial cell autoaggregation when expressed in *E. coli* K-12 (**Figure 4.3**). Interestingly, both tsh and sha gene expression was upregulated in the turkey respiratory tract (lungs and air sacs) further supporting a role for both proteins for infection of the lungs. The presence of both *sha* and *tsh* on a conjugative ColV plasmid support a potential for horizontal transfer to other

strains, and as these two plasmid-encoded SPATEs were predominant for respiratory infection, this may increase the likelihood of emergence of new APEC pathotypes if plasmids such as pEC598-01 are acquired in different *E. coli* isolates. Together along with the additional virulence traits encoded on such plasmids, their acquisition could potentially lead to novel types of ExPEC/APEC strains.

Although the other SPATEs– Vat, TagB, and TagC were not required for increased level of infection in turkey lungs, these SPATE –encoding genes were still expressed in the turkey lungs and air sacs. However, expression of *tsh* and *sha* were both highly upregulated in turkey lungs and air sacs (**Figure 4.6**). Nevertheless, Vat and TagBC might have other roles in colonization of other tissues or may also contribute to dissemination during systemic infection (Díaz *et al.*, 2020; Pokharel *et al.*, 2020).

In the turkey infection model, a striking finding was that the lungs were heavily colonized (10^7 – 10^8 CFU/gm of tissue) by either the wild-type QT598 strain, the 5-way SPATEs complemented Δ5SPATEs mutant, or the *tsh*+*sha* complemented Δ5SPATEs mutant. Notably, these 6-day-old turkeys were not significantly sick even though they had high levels of bacterial burden. The greater bacterial numbers in lungs may reflect tissue tropism or an increased capacity to resist host defenses. In vitro Sha, Vat and Tsh promoted biofilm formation and TagB, TagC and Sha also demonstrated autoaggregating phenotypes (Habouria *et al.*, 2019).

APEC strains are regarded as a potential foodborne zoonotic pathogen and a reservoir of extraintestinal infection in humans because of the genetic similarity between human ExPEC and some APEC strains (Bélanger *et al.*, 2011). Retail poultry meat are regarded as the possible route of exposure of humans to APEC carrying antibiotic resistance genes and other virulence genes contributing to ExPEC strains capacity to infect the urinary tract or cause neonatal meningitis (Johnson *et al.*, 2010b). It remains unclear why bacterial numbers were so elevated in the lungs of turkeys despite a relatively limited degree of disease present in the infected poults. It will be of interest to determine if such a phenotype may also be a potential means of transmission among poultry through respiratory route. Further, high level colonization by bacteria in the lungs may also increase the risk of contamination of the carcass and foodborne transmission to humans. From this standpoint SPATEs in addition to promoting respiratory infection in the turkey may also potentially promote transmission to humans by increasing the risk of contamination of poultry products from contaminated lungs and tissues following evisceration.

5 GENERAL DISCUSSION

Pathogenic *E. coli* possess a wide range of factors that contribute to virulence and host colonization. Toxins and proteases can contribute to virulence, and bacterial resistance to host immune defenses. Among these toxins, Serine Protease Autotransporters of *Enterobacteriaceae* (SPATEs) are important virulence factors of both InPEC and ExPEC (Pokharel *et al.*, 2019). *E. coli* QT598 which is an APEC O1: K1 strain encodes in total five distict SPATE proteins. These include two previously characterized SPATES, Vat and Tsh, and three newly described SPATEs TagB, TagC, and Sha. The *va*t and *tagBC* genes are located on the chromosome, whereas the *sha* and *tsh* genes are located on a ColV-type virulence plasmid (Habouria *et al.*, 2019). Multiple SPATEs might provide an advantage for virulence or colonization of different host niches through differential regulation, substrate recognition, or affinities during infection (Dutta *et al.*, 2002). It has been reported that CFT073 which is a UPEC strain contain 3 SPATEs: Vat, Sat and PicU (Parham *et al.*, 2004). *Citrobacter rodentium* and *Shigella flexneri* are also known to produce 3 SPATEs whose expression was shown to be differentially regulated (Bhullar *et al.*, 2015; Ruiz-Perez *et al.*, 2011). The five SPATEs present in strain QT598 were shown to have some shared and some distinct properties and differences in levels of expression in a mouse urinary tract infection model. The importance of these five SPATEs for potential urinary tract infection was established and characterized previously. The initial characterization of proteins and their competitive advantage to colonization of kidneys in mice demonstrated a collective importance of SPATEs for pathogenesis of QT598 (Habouria *et al.*, 2019). Based on these results, we were interested in investigating the role of SPATEs for QT598 in an avian air-sac infection model. Since QT598 was originally isolated from a turkey infection and was shown to be of low virulence in a chicken infection model, we opted to establish a turkey air-sac infection model. Overall, my thesis is focused on the establishment of a turkey air-sac infection model to study the possible functional hierarchy of five SPATEs for the colonization ability of strain QT598. We hypothesized that specific SPATE proteins alone or in combination could play an important role in colonization of host tissues and systemic infection for the pathogenesis of APEC in a natural host infection model.

5.1 Establishment of a turkey air-sac infection model

Initially, we investigated the potential of strain QT598 to infect the lungs, liver, spleen, and blood of turkeys and compared this to infection with an isogenic Δ*5SPATEs* mutant that had lost all 5 SPATEs by site-directed gene deletion. The SPATEs could play a role at different steps of infection including initial colonization or adherence and/or persistence through modulation of immune host defenses. SPATEs are known to confer multiple functions including adherence to host cells, agglutination, induction of vacuoles, formation of outer-membrane vesicles, motility, and biofilm formation (Kathayat *et al.*, 2021). The 5 SPATEs from QT598 contributed to colonization of the urinary bladder in co-infection in a murine UTI model, acted as cytotoxins, were internalized by epithelial cells and conferred adherence to epithelial cells (Habouria *et al.*, 2019; Pokharel *et al.*, 2020). QT598 was initially isolated from a young turkey poult (4-days old) and was virulent after subcutaneous injection in 1-day old chicks. However, both QT598 and the Δ5SPATEs mutant could only cause limited disease and was only present in reduced numbers from extra-intestinal organs and blood in either 21-day-old or 5-day-old chickens in the air-sac infection model. This poor capacity of strain QT598 to infect chickens could be due to a potential species-specific tropism to infect young turkeys. Host specificity of some types of APEC strains is further supported by the fact that, among 299 APEC strains that were screened, the *tagB* and *tagC* genes were only present in isolates obtained from infected turkeys (Habouria *et al.*, 2019). Further, in a recent report describing the novel P-like fimbriae (PLF) encoded adjacent to the *sha* autotransporter gene, screening of genomes positive for sequences specific to *plf* were more commonly isolated from turkeys and humans, but much less present in infections from chickens (Habouria *et al.*, 2022). From this we can infer that the air-sac infection model in young turkeys could more directly determine potential roles for SPATE genes in the virulence of APEC. Other examples of species specific *E. coli* pathotypes include EPEC strain, RDEC-1, isolated from a rabbit, that is unable to colonize the ileal and cecal regions of rats or guinea pigs, but which elicits severe diarrhea in rabbits (Rafiee *et al.*, 1991). In the same line, a human neonatal meningitis *E. coli* (NMEC) strain RS218 was shown to be less virulent in a duck infection model in comparison to a murine model, whereas by contrast an APEC strain DE471, isolated from duck brain, was more virulent in a duck model compared to a murine model (Zhang *et al.*, 2019). Altogether, these findings led us to establish a turkey air-sac infection model to study the role of SPATEs during colonization and systemic infection of different tissues during infection.

In the turkey air-sac infection model, two separate groups of 5-day-old turkeys were infected with 2.0×10^7 CFU per ml of the wildtype or the Δ5SPATEs mutant. The infection was by injection into the left air sac and allowed to proceed for 48 hours. During infection blood was collected and

plated for bacterial enumeration, however bacteria were not cultured from turkey blood at 6 h, 24 h and 48 h of infection. This suggests that strain QT598 is less able to persist in blood and may be less able to resist serum complement or host immune cells in the blood. After 48h, extra-intestinal tissues: the right lung, liver, spleen, and air-sac were collected to determine bacterial counts. Interestingly, we observed that the lungs infected with wildtype strains were heavily infected and colonized with 10^7-10^8 CFU per gram of tissue, whereas 5-ways SPATE mutants had 4-5 log fold lower bacteria in these tissues. However, the liver and spleen were not as heavily colonized as the lungs **(Figure 4.5).** This might signify the ability of strain QT598 to infect the respiratory system and demonstrate a tissue tropism for the lungs. Even with these high numbers of bacteria in lungs and pathogen burden in tissues in the turkeys, they did not show any strong clinical signs of disease when infected with either QT598 or the ∆5SPATEs mutant. However, turkeys infected with the ∆5SPATEs mutant significantly gained weight compared to those infected with the WT parent strain QT598. This difference may be attributed to the fact that SPATEs contributed to higher bacterial loads in tissues of turkeys infected with the wild-type strain and this resulted in some reduced appetite and recovery time when compared with the turkey infected with the ∆5SPATEs mutant which showed very limited signs of disease, were generally healthy in appearance gained weight. Altogether, we established that in the turkey air-sac infection model SPATEs were shown to be important for colonization and systemic infection of QT598 in some extra-intestinal tissues. However, such results did not ascertain if specific SPATEs could be of greater importance for colonization of turkey tissues during infection.

5.2 Analysis of expression of genes encoding SPATEs in the lungs and air-sacs of turkeys infected with strain QT598.

Another aspect of interest to investigate after establishing the air-sac infection model in turkeys was to determine if certain SPATE encoding genes were expressed at different levels in host tissues. We therefore determined expression levels of the 5 SPATE encoding genes in the lungs and air-sacs during colonization of the respiratory system. The levels of expression were determined by RT-qPCR from total RNA taken from lung and air-sac tissue samples. The cDNA was synthesized, and qPCR analysis was performed using primers specific for each of the five autotransporters. From qPCR analysis, we observed that the expression of *sha* and *tsh* were significantly higher compared to other SPATEs in the air sac and lungs of turkeys. These two genes are both encoded on the ColV-type virulence plasmid. This further supports the potential role of two ColV-plasmid encoded SPATEs as virulence genes for APEC and suggest that Sha and Tsh might play a more critical role for infection of turkey lungs by APEC strain QT598. Altogether, gene

expression analysis of SPATE-encoding genes provided insight of the possible hierarchy of the SPATE proteins during APEC pathogenesis.

5.3 Re-introduction of different SPATE genes to the ∆5SPATEs mutant to complement this strain and investigate a regain in APEC pathogenesis.

Using a bacterial genetics based approach of possible regain of function or phenotype through complementation can be undertaken to verify the principle of molecular Koch's postulates (Falkow, 1988). These postulates state that if a virulence trait is associated with the function(s) of a gene then inactivating that specific gene either by targeted or random mutagenesis should attenuate the virulence. The postulates further stipulate that the reintroduction of those specific genes into the mutant strain should restore the virulence trait (Falkow, 1988). As there are 5 SPATEs in strain QT598, the approach we favored was to delete all 5 SPATE encoding genes and then re-introduce some of the SPATEs to determine if certain genes may have a greater role in APEC virulence. The use of the mutant no longer producing any SPATEs was chosen as the best model to avoid potential issues of redundant compensatory functions due to the presence of multiple SPATE proteins. With the ∆5SPATEs mutant as our starting point, we aimed to reintroduce each SPATE gene or a combination of SPATE encoding genes to determine any hierarchical importance of specific SPATEs for extra-intestinal infection of turkey poults. Further, we also generated a complemented strain which had regained all 5 SPATEs to compare its capacity to infect with that of wild-type parent strain QT598.

A typical approach of complementing a mutation of a gene of interest is to reintroduce the mutated or deleted gene by cloning that gene into a recombinant plasmid (Caza *et al.*, 2011; Peterson *et al.*, 2003; Wang *et al.*, 1991). This approach can be an effective tool, particularly for *in vitro* studies where retention of the plasmid such as antibiotic selection can be included in the in vitro experimental model. However, selection pressure is not always suitable or feasible if experiments are to be done under in vivo conditions or in natural environments such as colonization or infection of live animals or fitness or survival of bacteria in environmental or food samples. In addition, even low-copy plasmids could cause an altered phenotype in complemented mutants due to the increase gene dosage compared to natively regulated expression levels in the wild-type parent strain. Further, in our particular model it would be problematic to try to re-introduce as many as 5 distinct genes using replicative multi-copy plasmid-based approaches (Crépin *et al.*, 2012a).

To avoid problematic issues associated with replicative plasmid-based complementation, we used a single-copy complementation through either allelic exchange recombination or a Tn7

transposase mediated insertion of functional SPATE genes into the bacterial chromosome. The Tn7 transposon integrates at the specific *att*Tn7 site in the chromosome which is located in the terminal loop of the conserved *glmS* gene (Peters *et al.*, 2001). A Tn7 system developed by the Dozois lab was used to this end (Crépin *et al.*, 2012a).

Using the aforementioned Tn7 complementation strategy, we re-introduced either the *vat, tsh, sha* or *tagBC* genes. In addition, we also re-introduced either the chromosomally encoded genes (*vat-tagBC*) or plasmid-encoded SPATE genes (*sha-tsh*) as separate combinations. Finally, in order to re-introduce all 5 SPATE genes to the quintuple SPATE mutant, both *sha* and *tsh* genes were introduced into the *lac* region (as described in (Porcheron *et al.*, 2014)) of the ∆5SPATEs mutant wherein the *vat-tagBC* genes had been introduced at the *att*Tn7 site. We thus generated a *lac*-negative derivative of ∆5SPATEs mutant that was complemented with all 5 SPATEs genes. We conducted turkey air-sac infection studies using these strains to determine to what extent either the presence of a particular SPATE or specific combinations of either chromosomally encoded or plasmid-encoded SPATEs restored the capacity to infect and colonize turkey tissues. To ascertain that each of the SPATE genes used for complementation were functional, we verified in vitro phenotypes such as auto-aggregation and oligopeptide cleavage activity. We also verified the clones for production and secretion of protein by SDS-PAGE and respective auto-aggregation ability. Further, for each of the complemented strains, we verified the presence of SPATE proteins in culture supernatants by Western blot and the activity of SPATEs were tested by using the oligopeptide cleavage assay. Altogether, we verified that complemented strains contained functional genes corresponding to each of the 5 specific SPATE proteins.

5.4 Reintroduction of all 5 SPATEs or only the *sha-tsh* plasmid-encoded SPATEs restored the infective capacity of the ∆5SPATEs mutant.

Air-sac infection with strains complemented with only the *sha, tsh, vat or tagBC* genes that were specifically reintroduced into the ∆5SPATEs mutant was not sufficient to restore the colonization ability of the strain in the lungs in comparison to wild-type parent strain QT598. Interestingly, when the ∆5SPATEs mutant was complemented with only *sha* or only *tsh,* the colonization levels increased when compared to the ∆5SPATEs mutant, although the levels were not significantly higher (**Figure 4.5**). Although the increase in bacterial numbers were not significant, these results suggested that the plasmid-encoded SPATEs could play a more predominant role in APEC colonization of turkey lungs during respiratory tract infection.

In fact, when both *tsh and sha* genes were reintroduced into the mutant strain, the strain gained significant colonization ability in the lungs to a level similar to that of wild-type strain QT598 (**Figure 4.5**). This supports the important role of these two SPATEs in infection of the turkey lungs. Further, Tsh and Sha both belong to the class 2 family of SPATEs and are also both located on a mobilizable ColV-type plasmid which carries many virulence genes including genes encoding the salmochelin siderophore (*iro* gene cluster), SitABCD metal transporter, HlyF, Increased serum survival (Iss*) ,and* aerobactin siderophore receptor *(*IutA*)* that have all been established to contribute to APEC virulence in certain strains (Reid *et al.*, 2022; Skyberg *et al.*, 2008). The additional presence of these 2 specific SPATEs on this ColV virulence plasmid further indicates that these transmissible plasmids can represent a major means of acquisition of multiple types of virulence factors through horizontal gene transfer.

Another interesting observation was that in the liver, the SPATEs mutant strain complemented only with *tagBC genes* had a regain in bacterial numbers comparable to that of wildtype parent strain. This suggests that the TagB and TagC autotransporters might have a more predominant role in infection of the liver. Since the tissue tropism of other systems is different than the lungs TagBC might play a selective role in other tissues such as the liver or tissues or environmental niches.

Finally, as a proof of principle of molecular Koch's postulates, re-introduction of all 5 SPATE encoding genes to the ∆5SPATEs mutant strain resulted in a regain in colonization levels in the lung, liver, and spleen of turkeys. Taken together, these results establish that the SPATEs are collectively important for the capacity of APEC strain QT598 to cause systemic infection in turkey poults. Although the regain of all 5 SPATEs was shown to be needed for full restoration of infectious capacity, we established that among the 5 SPATEs present in APEC QT598, the two ColV-type virulence plasmid-encoded SPATES *sha* and *tsh* were specifically more important for colonization of lungs during the systemic infection.

5.5 Relative levels of production of specific SPATEs proteins *in-vitro* in strain QT598 and SPATE mutant strains.

One of the intriguing questions concerning regulatory aspects of expression of the 5 SPATEs in strain QT598 is: "Which and how much of each SPATE is produced by a bacterial culture?" It would also be of interest to determine whether changes in growth conditions or other environmental cues or signals can result in changes in the levels of production of different SPATE proteins. Although, expression levels of gene transcription can be compared by transcriptome analysis or

experiments such as RT-qPCR, such approaches do not confirm what levels of SPATE proteins are secreted by bacteria during growth under different conditions. Further, it is also unknown whether the deletion or inactivation of certain SPATE encoding genes can alter levels of expression or production of the other SPATEs. To address such questions, SPATEs were obtained from culture supernatants following growth of parent strain QT598 and the different SPATE deletion mutants. From each of these samples, the relative abundance of each of the SPATE proteins was subjected to enzymatic digestion and peptides were analyzed by mass-spectrometry. With respect to the abundance of specific peptides unique to each of SPATEs, we observed that in wild-type strain QT598 that the relative abundance from highest to lowest levels of SPATES detected was Tsh, Vat, Sha, TagC, and TagB respectively, where Tsh was the most abundant among all SPATEs representing a total of 50% of all SPATEs present in supernatant samples. Interestingly, in the *tsh* mutant strain, the relative abundance of the Sha SPATE increased to 50% of the SPATES and Vat comprised 35% of the SPATE protein that was detected. In addition, in the *sha, tagBC and tagBC-vat* mutant strains Tsh was always the most highly produced SPATE. Further, in the absence of *tsh* and *sha* genes*,* Vat was the predominantly produced SPATE. These observations suggest that based on the in vitro culture conditions investigated, Tsh is hierarchically, the dominant SPATE produced by strain QT598. It would be of considerable interest to determine whether levels of gene expression under the same culture conditions compare to the proteomic data and to investigate if loss of certain SPATEs results in both a greater level of gene expression as well as protein production of certain SPATEs. In future work, it will be of interest to identify if there is regulatory crosstalk between expression of specific SPATEs encoding genes and to identify what regulatory mechanisms mediate the regulation and expression of different SPATE proteins in both APEC and other pathogenic *E. coli*.

6 CONCLUSION

In this thesis, we established the air-sac infection turkey model to address the role of SPATEs secreted by APEC as important virulence factors during the systemic colonization and infection through the respiratory tract. To do so we determined if molecular Koch's postulates were upheld by re-introduction of either individual or different combinations of SPATEs (Vat, Tsh, Sha, TagB and TagC) from a the ∆5SPATEs mutant derivative of APEC O1: K1 strain QT598. The ability of wildtype and mutant strains to infect the turkey lungs liver and spleen indicated that collectively the 5 SPATEs provided an important advantage for the strain to infect systemic tissues, since the ∆5SPATEs mutant was much less able to colonize any of these tissues. Moreover, since the re-introduction of all 5 SPATEs restored infectious capacity of all tissues to wild-type levels, this confirmed that collectively these SPATEs proteins contributed to the infectious capacity of APEC strain QT598. Furthermore, we investigated a potential hierarchical role of specific SPATEs for APEC pathogenesis. To do so we re-introduced SPATEs genes individually or a combination of subsets of these SPATE genes to the ∆5SPATEs mutant and tested their ability to infect host tissues. Results suggested that the plasmid-encoded SPATEs, Tsh and Sha individually could promote some colonization of lungs but at levels that were still significantly less than the wild-type parent strain. However, interestingly, the combined re-introduction of *sha* and *tsh* genes completely restored the ability of the mutant strain infect the lungs of turkey at levels similar to the wild-type parent. Further, re-introduction of the tandemly encoded *tagB* and *tagC* genes also provided some level of restoration of colonization in the liver. Moreover, the RT-qPCR analysis of bacterial RNA from lungs and air-sacs of turkeys infected with strain QT598 indicated that *tsh* and *sha* genes were more highly expressed during infection than the other SPATE encoding genes. Further, mass-spectrometry analysis of the secreted SPATE proteins produced *in-vitro* in iron restricted minimal media at 41 degrees Celsius also demonstrated a relatively higher abundance of Tsh and Sha proteins compared to the three other SPATEs. Altogether, these results establish that the SPATES Tsh and Sha, encoded by Col-V type virulence plasmid pEC598-1, are at the peak of the hierarchical role of the five SPATEs that contribute to systemic infection of the turkey by avian pathogenic *E. coli* (APEC), QT598 O1: K1 strain.

This study represents one of the first uses of a turkey infection model to investigate APEC pathogenesis and to attempt to pinpoint specific roles of a set of virulence proteins that could possibly play redundant or overlapping roles in infection. In future work it will be of interest to identify regulatory mechanisms underlying expression of SPATE proteins more specifically. Since the

SPATEs proteins are known to have multiple effects on host cells and modulation of immune defenses, it will also be of specific interest to investigate the effects of specific SPATES on host immune function both in avian as well as human cells, since these different SPATEs can be found both in ExPEC strains causing infections in both humans as well as poultry. Several different SPATEs or autotransporters including multiple distinct genes have also been identified in other *E. coli* pathotypes or bacterial pathogens. As such, the approaches we have implemented herein combining molecular Koch's postulates, single-copy gene complementation, transcription analysis and proteomics, could also be applied to determine hierarchical roles of SPATEs in other *E. coli* pathotypes or other bacterial pathogens in different types of infection disease models.

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