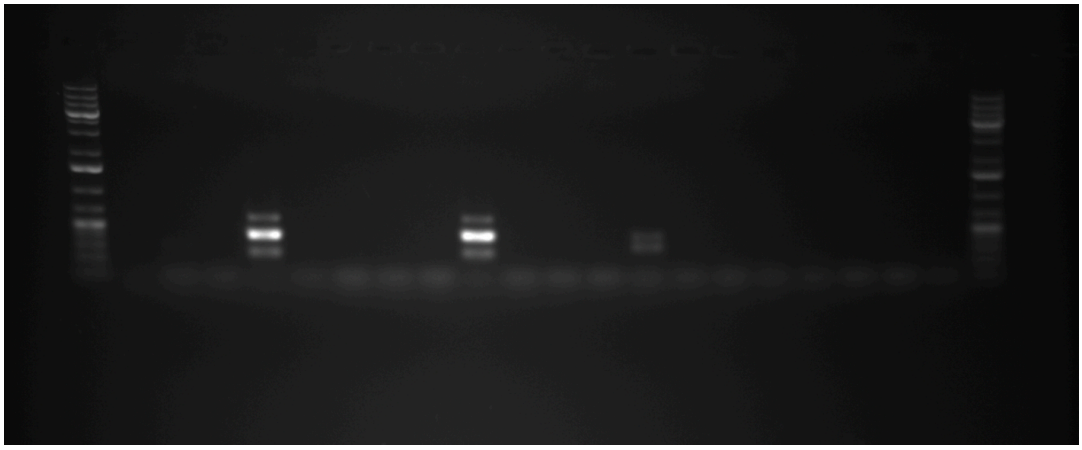
ROSKILDE UNIVERSITY

INSTITUTE OF NATURAL SCIENCE AND ENVIRONMENT

Detection of Enteroaggregative *E. coli* virulence genes in ulcerative colitis patients



BACHELORPROJECT IN NATURAL SCIENCE

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Preface

This bachelor project is developed by Selin and Katrine from the natural science bachelor department in Roskilde University at the spring semester 2020. The project is aimed for people with a background in molecular biology and microbiology.

This project is a continuation of our 4th semester project," Investigating *possible receptor binding pocket of E. coli adhesin"*, where the function of a specific fimbriae variant responsible for host colonization and transmission by Enteroaggregative *E. coli* (EAEC) were investigated. In this project we want to investigate if there is a coherence between patients with Ulcerative Colitis (UC) and the presence of EAEC in the intestinal of UC patients. The project was planned to be an experimental, but because of the Covid-19 situation, the entire experimental part was not performed. Instead we have PCR results run on gel and phylogenetic data from Hengameh Chloe Mirsepasi-Lauridsen, which we discussed with relevant articles conducting similar experiments.

In relation to writing the dissertation we would like to express our gratitude to Hengameh Chloe Mirsepasi-Lauridsen, who has contributed with guidance, knowledge as well as practical activities and stool samples for testing in the laboratory. Further, we want to thank Yasemin Karatas for education and instruction in cell splitting and Kirsten Olesen for her help in the laboratory. At last, we are grateful to our supervisor Karen Angeliki Krogfelt, for her support through the whole project.

The reference system used for this project is Mendeley

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Roskilde, May 2020.

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Abstract

Ulcerative colitis (UC) is a chronic inflammatory bowel disease of the mucosal surface which is identified by persistent diarrhea, abdominal pain and bleeding from colon. The pathogenesis of UC is still not fully understood, and the bacteria-dysbiosis are linked to disease relapses. Especially virulent *E. coli* is elevated in stool samples of UC patients. The diarrheagenic of Enteroaggregative *Escherichia coli* (EAEC) are frequently found in stool of patients with diarrhea, hence our aim is to investigate the presence of EAEC in stool sample of UC patients and clarify if there is an association between EAEC and active UC. A multiplex PCR targeting *aap, AggR, aaiC* and *aatA* are performed to detect the virulence genes for EAEC. The study includes 100 patients with 185 *E. coli* isolates. 9% of the patients had at least one of the above mentioned EAEC virulence genes. Further experiments are needed to confirm the link between EAEC and UC disease activity, including investigating prevalence of phenotyping and serotyping of EAEC in active UC.

Resume

Colitis ulcerosa er en kronisk inflammatorisk tarmsygdom der forårsager inflammation på slimhindens overflade. Den kendetegnes ved vedvarende diarré, mavesmerter og blødning fra kolon. Patogenesen af colitis er stadig ikke fuldstændig klarlagt dog er bakterier linket med inflammationen. Der er specielt fundet forhøjet mængder af *E. coli* i colitis patienters afføringsprøver. De diarrefremkaldende Enteroaggregative *Escherichia coli* (EAEC) er hyppigt detekteret i diarre patienter. Derfor ønsker vi at undersøge tilstedeværelsen af EAEC i afføringsprøver fra colitis patienter og deraf klarlægge om der er en association mellem EAEC og aktiv colitis. En multiplex PCR, der er målrettet mod *aap, AggR, aaiC* og *aatA*, udføres for at påvise tilstedeværelsen af virulensgenerne for EAEC. Undersøgelsen omfatter 100 patienter, med 185 *E. coli* stammer. I 9% af patienterne blev der detekteret en eller flere af virulensgenerne. Der bør udføres flere eksperimenter for at bekræfte tilstedeværelsen af EAEC i UC-patienter med inflammation, herunder forekomst af specifik EAEC fænotypning og serotypning i disse patienter.

Ulcerative Colitis – The incurable Bowel disease

Written by Katrine Gry Gulløv (kgryg@ruc.dk) and Selin Arife Yüksel (say@ruc.dk)

Chronic Inflammatory Bowel disease (IBD) is an increasing problem in the developed countries especially in northern Europe, North America, Canada and Australia. In a report from "*Rådet for Anvendelse af Dyr Sygehusmedicin*" (RADS), it is assumed that around 45.000 Danish people suffers from chronic IBD and approx. 30.000 out of those suffers from Ulcerative Colitis (UC). In addition, there are approx. 850 new incidents of UC every year in Denmark. IBD is an autoimmune disease that leads to intestinal inflammation of the gastrointestinal tract (GI). It is characterized by periods of remission and relapse. IBD has traditionally been divided into Crohn's disease (CD) and ulcerative colitis (UC), mainly by the location in the intestine. CD can be found in most of the GI tract while UC effects only rectum and colon, they are both multifactorial diseases, but the exact cause is unknown. However, the key features are aberrant immune response, genetic predisposing, changes in the intestinal microbiota and environmental factors. In addition, previous studies indicate that virus or bacterial infections seems to trigger the diseases, especially *Escherichia coli* (*E. coli*) is suspected to play an important role. There has been found an increased amount of *E. coli* in the intestine of IBD patients compared to healthy individuals. There is no cure

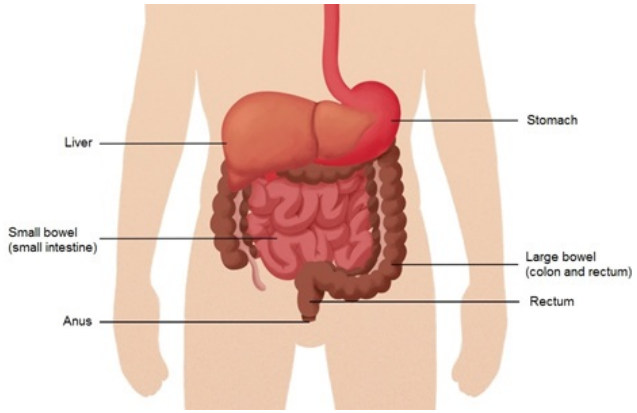
for IBD, and medical therapy is only given to obtain and remain remission and prevent hospital admission and surgery.

There is a lot of research in IBD to understand the diseases and to make life more comfortable for the patients, but much is still unclear. In this article we

focus on UC. We are two bachelor students from Roskilde University who has investigated stool samples from 100 UC patients to see if we can identify a specific diarrheagenic *E. coli,* the Enteroaggregative *Escherichia coli* (EAEC). EAEC is the most common bacteria found in patients with persistent diarrhea. We have investigated if there is a coherence between UC and the presence of EAEC in the intestine of UC patients.

Ulcerative Colitis

UC is an inflammatory bowel disease that effects rectum and colon. It is identified by persistent diarrhea, abdominal pain and bleeding from colon and rectum. The precise cause of the disease is unclear, but there are several independent risk factors. First, there is genetic predisposing, the risk of developing UC is increased in first degree relatives. Second, there are environmental factors, there are prevalence of UC in developed countries. This indicates that the improved sanitation and the increased access to healthcare could cause a decrease in intestinal infections throughout the childhood, that leads to aberrant immune response in the intestine. Diet also seems to have an influence on the composition of the intestinal microbiota early in life. Studies show that a high daily intake of fast food increases the risk of UC. It is a uncurable disease and the medical therapy is only given to improve the quality of life for the patients.



Illustrates the Gastrointestinal tract. Colon and rectum are shown in the figure, which is affected in UC patients.

Enteroaggregative *Escherichia coli*

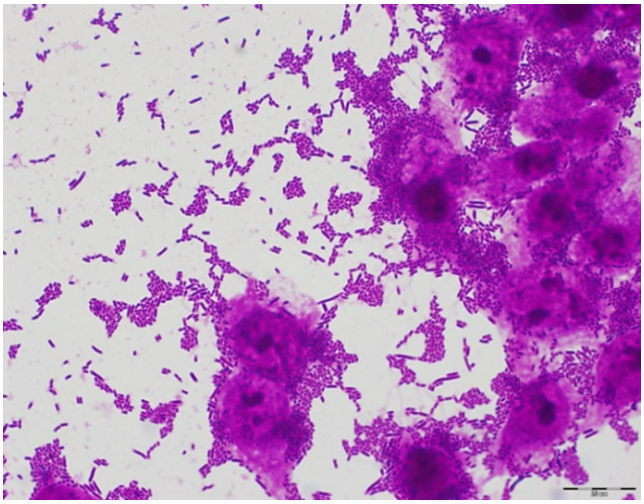
E. coli is a commonly found bacteria in the GI tract of humans, where it most often lives in symbiosis with the host. At the same time, it is the most frequent cause of diarrhea worldwide. This is among others, caused by *E. coli's* ability to exchange genetic material with other bacteria. It makes it a very heterogeneous bacterium, so classification has been necessary.

Classification is done according to the bacteria's properties and genetics. *E. coli* that causes disease is called pathogenic and they are divided into two groups: The *E. coli* causing diarrhea in the GI tract are known as diarrhoeagenic *E. coli* (DEC) and the *E. coli* causing disease outside the GI tract are called extraintestinal pathogenic *E. coli* (ExPEC). ExPEC is responsible for urinary tract infection (UTI), sepsis and meningitis.

Enteroaggregative *Escherichia coli* (EAEC) is a DEC and the most common bacteria identified in stool samples from patients with persistent diarrhea. EAEC is characterized by the ability to adhere to mucosa cells in the intestine and make a characteristic "stacked-brick" pattern. It is responsible for acute and persistent diarrhea in children in developing countries and immune-compromised patients as well as travelers' diarrhea. EAEC is very heterogeneous and several virulence genes are identified. Moreover, some EAEC strains has acquired ExPEC genes which makes them responsible UTI and sepsis.

The investigation

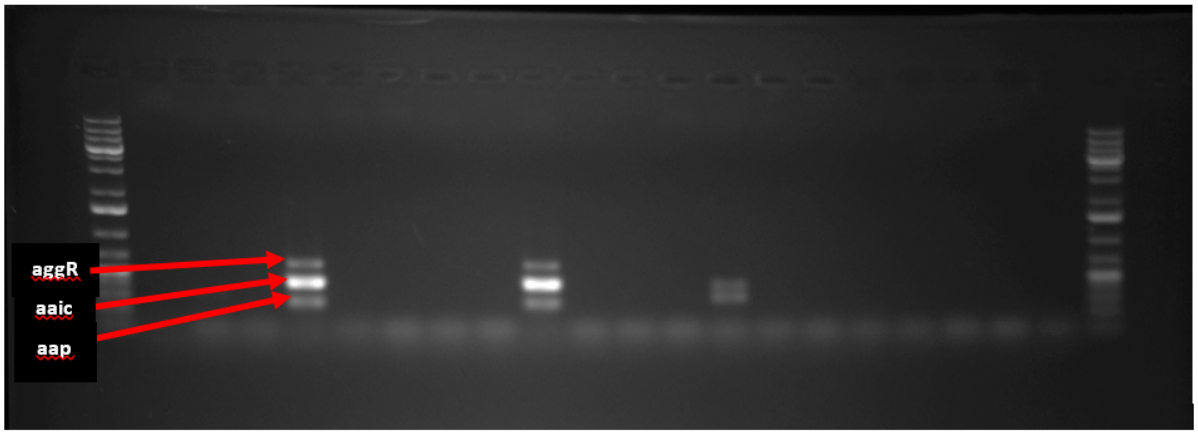
Research in UC is important when trying to understand the disease and for a more determined treatment. In our study, we have investigated



The characteristic "stacked-brick" pattern of adherence to human mucosa cells in the intestine. The darkest spots are the cells and the small rod shape is the E. coli bacteria.

185 stool samples from 100 UC patients to see if we could identify a coherence between UC and EAEC. The purified stool samples were tested for 4 different virulence genes *aap, aaiC, aggR* and *aaiC* which is characteristic for EAEC.

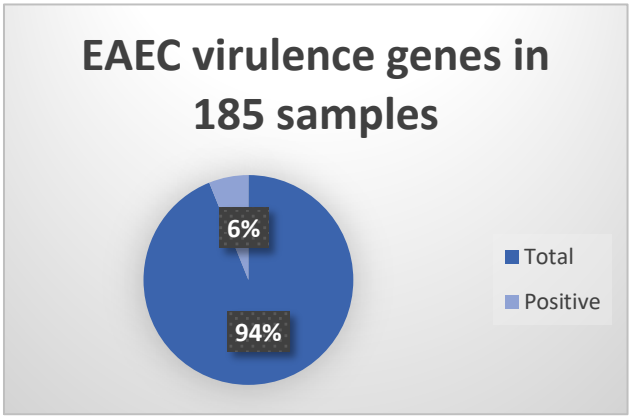
By using Polymerase Chain Reaction (PCR), we multiplied the genes if they were present in the sample. Afterwards, we separated them by size, using gel electrophoreses. The sample were stained and visualized under ultraviolet light.

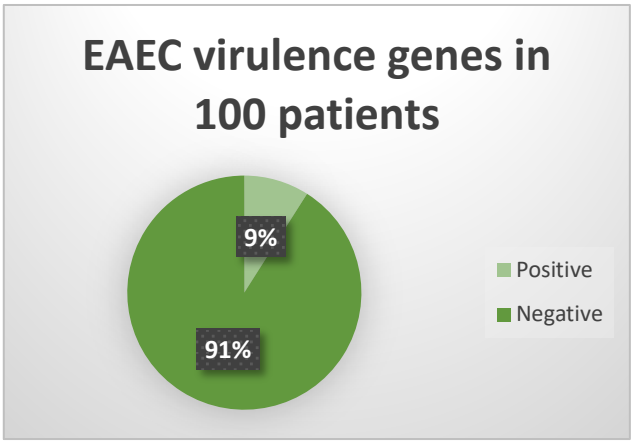


a picture of one of our gel electrophoreses. On each side you see a size marker. In three of the samples in the picture, there are respectively two or three genes visualized.

The results

The result shows that 6% of the samples contains one or more of EAEC virulence genes equivalent to 9% of the patients.





Maybe it does not sound like much, but taken into consideration that the human intestinal microbiota consist of a great variety of bacteria, it must be considered as a strong indication on a coherence between UC and EAEC. Samples with three virulence genes is most likely EAEC, but sampels with only one is more doubtful due to the fact that *E. coli* exchange genetic material with other bacteria or strains of *E. coli*. Therefore further investigation is needed. The next step would be to make a genetic analysis of the positive samples and a phenotyping. Phenotyping is the investigation of the visual proberties of an organism. Here we would observe if our positive samples makes the characteristic "stacked-brick" pattern to human cancer cells. Other studies have investigated the coherence between IBD and different groups of *E. coli*. In an Egyptian study they found EAEC in 25% of the samples from IBD patients. The result is

associated with uncertainties, since they only tested for one
gene. We cannot expect the same result in Denmark, hence there are different environmental factors, such as sanitation, diet and antibiotic consumption. Several other studies have found coherence between IBD and other DEC, but much more research is needed for a better understanding of the disease and thus a better treatment of the patients.

For further reading:
https://rads.dk/media/4294/bgn-gast-237153-merged.pdf

https://pubmed.ncbi.nlm.nih.gov/30700431/

file:///C:/Users/KG/AppData/Local/Packages/Microsoft.MicrosoftEdge_8wekyb3d8bbwe/TempState/Downloads/Enteroaggregative_Escherichia_coli_EAEC%20(1).pdf

https://pubmed.ncbi.nlm.nih.gov/32042723/

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List of Abbreviations

AAF: Aggregative Adhesion Fimbriae

AIEC: Adherent invasive *Escherichia coli*

CD: Crohns disease

DAEC: Diffusely Adherent *Escherichia coli*

DEC: Diarrheagenic *Escherichia coli*

EAEC: Enteroaggregative *Escherichia coli*

EHEC: Enterohemorrhagic *Escherichia coli*

EcN: *Escherichia coli Nissle*

EIEC: Enteroinvasive *Escherichia coli*

EPEC: Enteropathogenic *Escherichia coli*

ETEC: Enterotoxigenic *Escherichia coli*

ExPEC: Extraintestinal pathogenic *Escherichia coli*

HEp-2: Human epithelial 2 cells

IBD: Inflammatory bowel disease

PAI: Pathogenicity island

PCR: Polymerase Chain Reaction

Pet: Plasmid encoded toxin

SepA: Shigella extracellular protein A

SPATES: Serine protease autotransporters of *Enterobacteriaceae*

STEC: Shigatoxin-producing *Escherichia coli*

TNF-α: tumor necrosis factor alpha

UC: Ulcerative Colitis

UTI: Urinary Tract infections

5-ASA: 5-aminosalicylic acid

Introduction

Chronic Inflammatory bowel disease (IBD) is an increasing problem in the developed countries especially in northern Europe, North America, Canada and Australia. In a report from "*Rådet for Anvendelse af Dyr Sygehusmedicin*" (RADS), it is assumed that around 45.000 Danish people suffers from chronic IBD and approx. 30.000 out of those suffers from Ulcerative Colitis (UC). In addition, there are approx. 850 new incidents of UC every year in Denmark (Jens Kjeldsen et al. 2016). IBD can be divided into two main groups, Crohn's disease (CD) that induces inflammation in the gastrointestinal tract and Ulcerative Colitis (UC) which is a chronic inflammatory disease that effects rectum and colon. We have delimited this dissertation to primarily focusing on UC, and Crohn's disease will only be mentioned in relations to underlining common denominators. UC is identified by persistent diarrhea and rectal bleeding. It is a multifactorial disease, but the exact cause of the disease is unknown. However, the key feature is aberrant immune response, genetic predisposing, changes in the intestinal microbiota/dysbiosis and environmental factors (Ungaro et al. 2017a). Furthermore, several studies indicate that virus or bacterial infections could trigger UC, especially *E. coli* suspected to play a role in the pathogenesis (Hengameh Chloé Mirsepasi-Lauridsen et al. 2019; Andreas M. Petersen et al. 2009). Enteroaggregative *Escherichia coli* (EAEC), a subgroup of the diarrheagenic *E. coli* (DEC), is found to be the most common bacterial pathogen identified in diarrheal stool samples (Croxen et al. 2013). EAEC is identified by its ability to make the characteristic "stacked-brick" pattern of adherence to human epithelium. It is responsible for acute and persistent diarrhea in children in developing countries and in immunocompromised patients as well as travelers' diarrhea (Elias and Navarro-Garcia 2016). In 2011 EAEC was directly linked to a diarrhea outbreak in Germany that infected 3910 people and resulted in the loss of 46 human lives (Cheung et al. 2011).

Aim of The Project

The aim of the project is to investigate the presence of EAEC in stool samples from ulcerative colitis patients with active disease, to see if there is a correlation between active UC and EAEC.

Background

Ulcerative colitis

UC is an inflammatory bowel disease that affects colon and rectum. It is a chronic inflammatory disease of the mucosal surface which is identified by persistent diarrhea, abdominal pain and bleeding from colon (Ordás et al. 2012). UC normally starts in the rectum and extents to the proximal segment of the colon. It is characterized by periods of remission and relapse. Patients can be classified into different subgroups based on location and spread of the infection in the intestine (figure 1). Most patients 30-60% diagnosed with UC have proctitis, where inflammation occurs in rectum. The symptoms are rectal bleeding, tenesmus and urgency. 16-45% have left-sided colitis, with symptoms as bloody diarrhea and abdominal cramping. 14-35% have extensive pancolitis, which includes inflammation in most of colon and rectum. The symptoms are like left-sided colitis, but with additional symptoms such as fever, fatigue and weight loss due to lack of appetite and lost ability to absorb nutrients. 10-19% of the patients will experience a progress in the state of disease after 5 years and up to 28% after 10 years (Ungaro et al. 2017a).

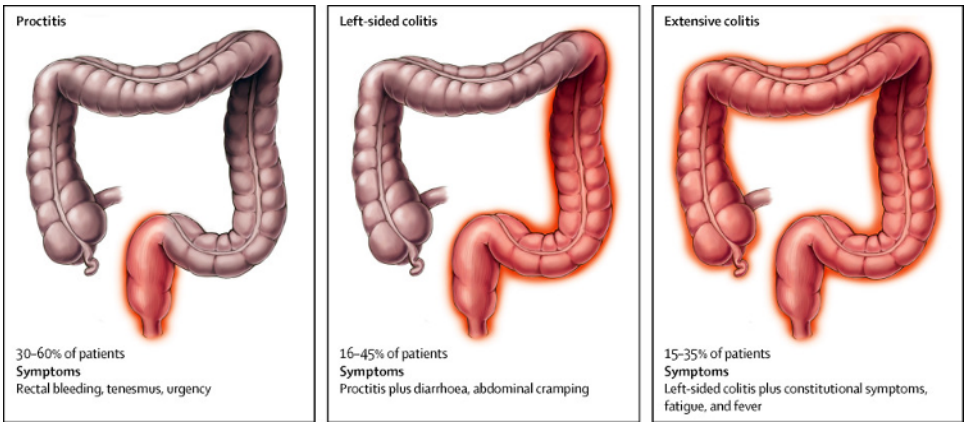

Figure 1 illustrate the location and spread in the three different subgroups of UC, proctitis, left-sided colitis and extensive colitis. (Ungaro et al., 2017)

The pathogenesis is multifactorial, and the precise cause of UC is unclear. However, there are several independent risk factors (Ungaro et al. 2017a). First, there is genetic predisposing, the risk of developing UC is increased in first degree relatives (Childers et al. 2014). Second, there is an environmental factor, there is prevalence of UC in developed countries. The incidence of UC is highest in north Europe, Canada, North America and Australia. Therefore, it is suggested that the improved sanitation and the increased access to healthcare could cause a decrease in intestinal infections

throughout the childhood, that leads to an aberrant mucosal immune response. Furthermore, diet seems to have an influence on the composition of the intestinal microbiota early in life. Studies show that a high daily intake of fast food increases the risk of UC. It seems to result in a homeostatic unbalance between the mucosal immunity and the enteric microflora in UC patients (Bernstein et al. 2006; Hengameh Chloé Mirsepasi-Lauridsen et al. 2019; Ordás et al. 2012). The onset of disease can be at all ages with no sex preference, but with a peak age at 30-40 years (Ungaro et al. 2017a). There is registered several different aberrant immune responses associated with UC, but a very important factor is the homeostatic unbalance between regulatory and effector T-cells. An atypical T-helper cells (Th-2) response mediated by macrophages and dendritic cells, activates T-killer cells to excrete interleukins IL5 and IL13. Especially IL13 is of great importance, because it is responsible for excretion of cytotoxins, which function against epithelial cells, including inducing apoptosis and alteration of the protein structure of tight junction. This leads to an increased permeability in the epithelium and enable uptake of luminal microbes and antigens which might cause inflammation (figure 2) (Ordás et al. 2012).

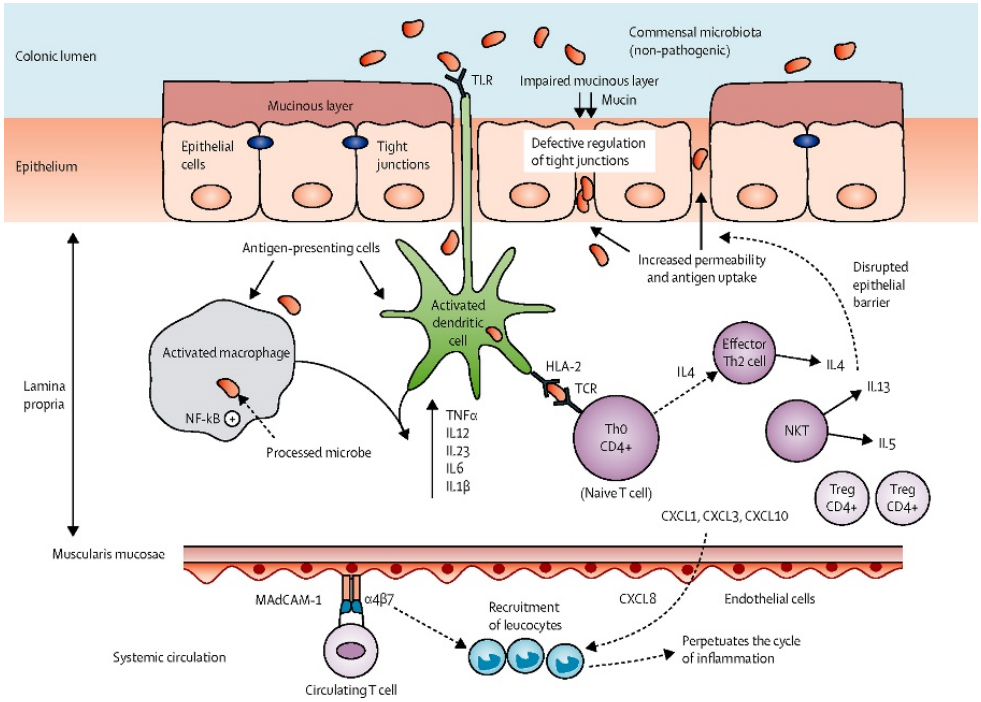

Figure 2 Commensal microbiota in the colonic lumen is recognized by macrophages and dendritic cells and presents them for the native CD4 cell. CD4 cells activates the Effector Th2 cells, that excrete interleukin 4 (IL4). The natural-killer T-cells is activated by IL4 to secretion of IL5 and IL13. IL13 reacts against the epithelium and induces apoptosis and alteration of the protein structure of the tight junctions. This leads to an increased permeability of the epithelium and enable uptake of more commensal microbiota which causes infection (Ordás et al., 2012).

Knowledge about the disease pathophysiology of UC is important for developing the best treatment strategies. The aim of the treatment strategies is that patient obtain and remain remission and prevent hospital admission, surgery and colorectal cancer. The treatment consists mainly of 5-aminosalicylic acid (5-ASA), corticosteroids, immunosuppressive drugs and anti TNF-α drugs (also known as biologic agent). 5-ASA drugs are used as first-line treatment for mild to moderate UC and is administrated as suppositories, enemas or oral formulations. If patients do not respond to 5-ASA drugs, corticosteroids can be used as second-line add-on treatment to induce remission. In later stages of the disease, a combination treatment is applied. The treatment strategies are individualized for each patient according to age, symptoms, stage of the disease and comorbidities. For 20-30% of the patients medical therapy is not successful and surgery may be considered (Ordás et al. 2012; Ungaro et al. 2017b). The risk of developing colorectal cancer is increased with the years and it is estimated to be 2% after 10 years, 8% after 20 years and 18% after 30 years of disease (Lakatos and Lakatos 2008).

UC and *E. coli*

E. coli is suspected to play an important role in the onset of IBD and several studies have found an increased quantity of *E. coli* strains containing virulence properties in IBD patients compared to healthy individuals (Kotlowski et al. 2007; Andreas M. Petersen et al. 2009). Especially, Adherent invasive *E. coli* (AIEC) has been associated with CD while diffusely adherent *E. coli* (DAEC) has been isolated from UC patients with both enterotoxigenic and enteropathogenic properties (Darfeuille-Michaud and Colombel 2008; Andreas M. Petersen et al. 2009). In addition, treatment with antibiotic has shown to improve the condition of IBD patients and initiate remission. Interestingly, genetic analyses of the *E. coli* strains isolated from stool samples and biopsy from IBD patients shows that most of the *E. coli* strains belonging to the phylogenetic group B2, which normally includes extraintestinal pathogenic *E. coli* (ExPEC) (Hengameh Chloé Mirsepasi-Lauridsen et al. 2019; Andreas M. Petersen et al. 2009).

Enteroaggregative *Escherichia coli*

E. coli is non-pathogenic bacteria in the human intestine. The bacteria can acquire some pathogenic factors and can cause illness such as diarrhea. The diarrheagenic *E. coli* (DEC) is divided into 6 sub-groups: enteropathogenic *E. coli* (EPEC), enterohemorrhagic (Shiga toxin-producing) *E. coli* (EHEC/STEC), enteroaggregative *E. coli* (EAEC), enterotoxigenic *E. coli* (ETEC), and enteroinvasive *E. coli* (EIEC) (Gomes et al. 2016; James P. Nataro and Kaper 1998).

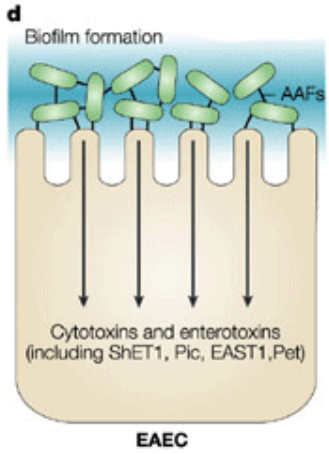

Figure 3 EAEC colonizing the epithelial layer and creating biofilm. Cytotoxins and enterotoxins are released into the epithelial cells. Modified from (Kaper, Nataro, and Mobley 2004)

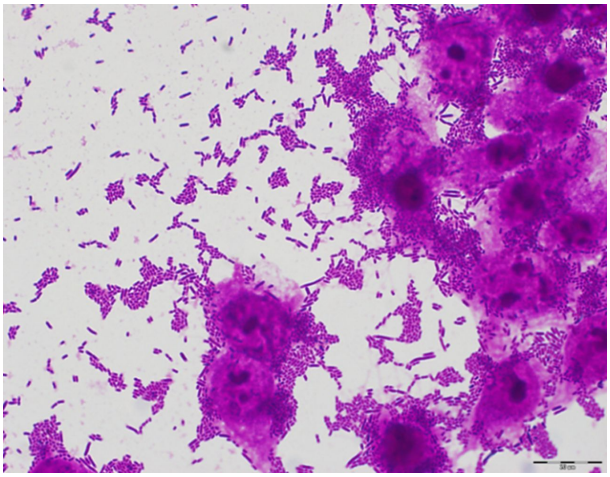

Figure 4 – EAEC characterized stacked brick pattern on Hep-2 cells under a microscope after staining. Courtesy of Rie Jønsson, Department of Microbiology and Infection Control, Statens Serum Institut, Copenhagen, Denmark

EAEC is a common cause of diarrhea and is associated with acute and persistent diarrhea worldwide (Jønsson et al. 2017; J P Nataro et al. 1992). EAEC is characterized with its stack-brick pattern on HEp-2 cells (figure 4). It is very heterogenous and can have several virulence factors contributing to the inflammation of the epithelial layer. All the virulence factors are not present in all the strains and the characteristic stacked brick pattern does not always occur which makes the identification of EAEC challenging (Jønsson et al. 2015). EAEC attaches to the cell mucosa with its aggregative adhesion fimbriae (AAF) and it creates a biofilm layer, which makes diffusion of cytokines and enterotoxins possible (figure 3) (Kaper, Nataro, and Mobley 2004). Several studies indicate that the AAF has a major impact on the adhesion to the mucus layer and it is essential for the inflammation (Hicks, Candy, and Phillips 1996; Jønsson et al. 2017). The EAEC virulence genes are also found in *E. coli* isolates from Urinary tract infections (UTI) which indicate that EAEC also can be categorized as an ExPEC which causes UTI (Boll et al. 2013).

Pathogenesis and virulence factors

The pathogenesis of EAEC occurs in three steps: adherence, toxin release and inflammation. The adherence is mediated by the AAF and it is suggested to have a major impact on the pathogenesis (Hicks, Candy, and Phillips 1996). Furthermore, a thick layer of biofilm is formed by the EAEC, which is proposed to make a barrier against antibiotics and antimicrobial factors (Sheikh et al. 2001). Several virulence factors contribute to the EAEC pathogenesis. The AAF is regulated by the AggR regulator and also regulates the Aat transporter system which is suggested to transport the dispersin out of the bacterial cell (Nishi et al. 2003). The dispersin is encoded in the *aap* gene and it is a colonization factor which promotes the dispersal of the cells and penetration of the mucus layer (Sheikh et al. 2002). Toxins are released, which is damaging the intestinal barrier and causing inflammation (see figure 5). The toxins Pet, Pic, SepA belonging to the Serine protease autotransporters of *Enterobacteriaceae* (SPATES) are released. Other toxins (see table 1) such as EAST-1 and shET1 is also

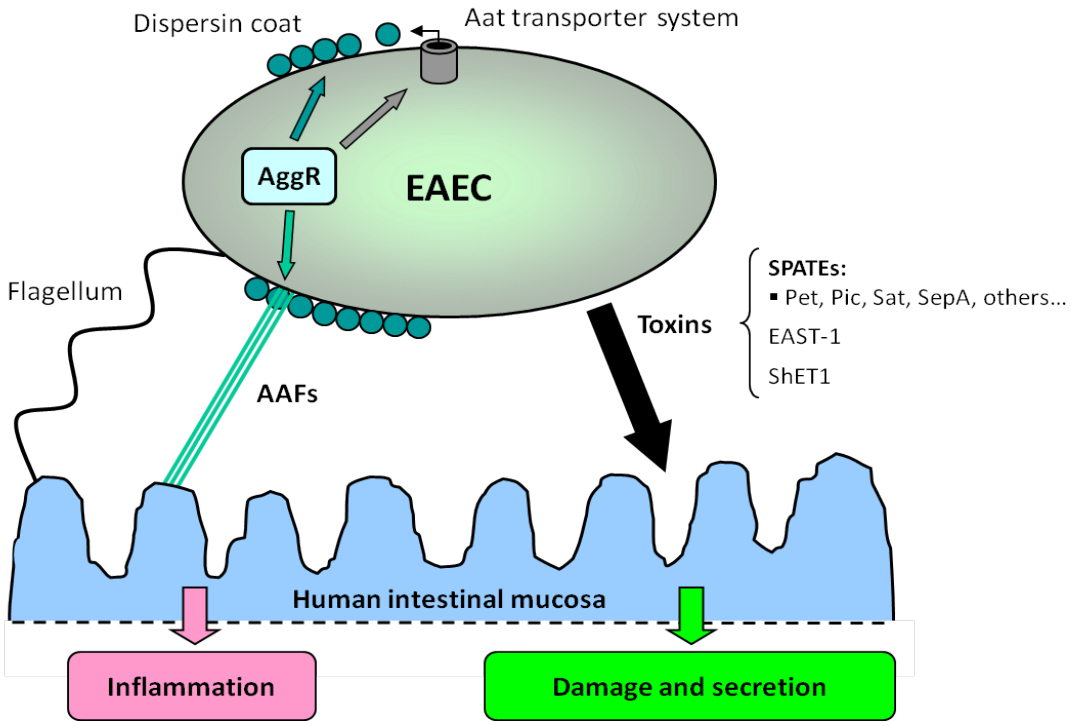

Figure 5 Pathogenesis of EAEC. The AggR regulates the dispersin coat, the Aat transporter system and the AAF's. AAF adhere to the Human intestinal mucosa. Enterotoxins and cytotoxins damage the epithelial cell which causes inflammation (James P. Nataro 2005). Further description in the text. Courtesy of Erik Juncker Boll

released. Pet (Plasmid encoded toxin) is using the V secretion system and it causes disruption in the actin cytoskeleton (Navarro-Garcia 2010). The *pic* gene is associated with diarrhea and *pic* is causing diarrhea independently in the presence or absence of other genes (Durand et al. 2016). Shigella extracellular protein A (SepA) is facilitating invasion of the epithelial layer, and this toxin is also associated with diarrhea (Boisen et al. 2012; Maldonado-Contreras et al. 2017). The toxin EAST-1 is encoded in the *astA* gene and its pathogenesis and virulence is not clear except for its association with diarrheal diseases (Dubreuil 2019; Mendez-Arancibia et al. 2008). Common EAEC virulence factors are listed in table 1 with a description of the EAEC factors and locations.

Table 1. Overview of genes and toxins found in the EAEC pathotype with description and location (Jenkins 2018).

Table 1 Genes and toxins often found in the EAEC pathotype

Common EAEC factor	Description	Location
aggR	Master regulator for EAEC plasmid virulence genes, including aggregative adherence factors, fimbriae AAF/I-AAF/V, and a large cluster of chromosomal genes inserted on a pathogenicity island at the PheU locus	pAA
aatA-P	Encodes proteins responsible for transporting the dispersin protein out of the outer membrane of EAEC	pAA
aap	Encodes a 10 kDa secreted protein named dispersin and is responsible for "dispersing" EAEC across the intestinal mucosa	pAA
aggA	Encodes AAF/I mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
aafA	Encodes AAF/II, mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
agg3A	Encodes AAF/III haemagglutination of erythrocytes	pAA
agg4A	Encodes AAF/IV mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
agg5A	Encodes AAF/V mediates adherence to colonic mucosa and haemagglutination of erythrocytes	pAA
aaiA-Y	PAI encoding a type VI secretion system (T6SS)	chromosome
pet	A 108 kDa autotransporter protein that functions as a heat-labile enterotoxin and cytotoxin	pAA
sigA	IgA protease-like homologue, enterotoxin and cytotoxin	Chromosome
pic	Mucinase, immunomodulation, colonisation, lectin-like haemagglutinin	Chromosome
sepA	*Shigella* extracellular enterotoxin	pAA
sat	Secreted autotransporter toxin. Enterotoxin and cytotoxin, impairment of tight junctions, autophagy	pAA
astA	*astA* encodes the enteroaggregative heat-stable toxin (EAST-1), which has physical and mechanistic similarities to *E. coli* STa enterotoxin	pAA

Experiments and Methods

Methodology

The background and knowledge about the patients are from the article *"Ciprofloxacin and probiotic Escherichia coli Nissle add-on treatment in active ulcerative colitis: a double-blind randomized placebo controlled clinical trial."* (Andreas Munk Petersen et al. 2014). The same stool samples are analyzed in this study.

By performing a multiplex-PCR designed to detect the specific EAEC virulence genes *aap, aaiC, AggR* and *aatA*, we will be able to confirm the presence of EAEC in *E. coli* isolated from feces of UC patients with active disease. The PCR product will be loaded on an agarose-gel and depending on the size of the fragments we can verify present of the correct EAEC genes in isolated *E. coli*. The stool samples are form patients prior entering an intervention treatment study (thus without any treatment given). If the PCR confirms the presence of the virulence genes, we supposed to perform serotyping and detect surface antigens. Furthermore, the characteristic stacked-brick pattern was supposed to be observed by adherence assay with Hela-cells.

Patients

The study in the article *"Ciprofloxacin and probiotic Escherichia coli Nissle add-on treatment in active ulcerative colitis: a double-blind randomized placebo controlled clinical trial."* is conducted on totally 100 UC patients with active disease. Figure 6 shows an overview of the patient's gender, disease extension and their medical history with treatments before the study are listed. 38 of the patients are men and 62 are women where most of the patients have left-sided UC. The medical history for the majority is treatment with systemic prednisolone and Azathioprine/6-mercapto-purine. All the patients are aged >18 years and patients currently treated with systemic corticosteroids or biologic therapy were excluded. The patients were followed for 12 weeks and at the end of the experiment 74 patients were left in the study. This is due to the lack of remission in patients which necessitated treatment and some patients wished premature termination from the study.

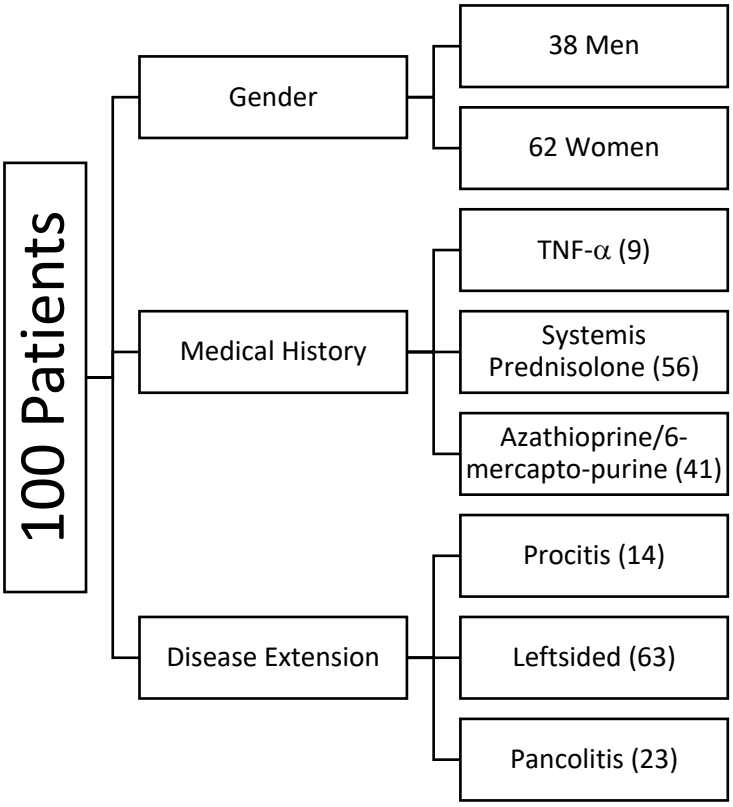

Figure 6 Chart over the patients included in the study by (Andreas Munk Petersen et al. 2014)

Polymerase Chain Reaction (PCR)

The determination of the EAEC genes in the UC samples will be investigated by performing a multiplex-PCR where the genes are *aap, aaiC, aggR* and aatA are targeted (Boisen et al. 2012). The *aap* encodes the dispersin protein which is located on the pAA plasmid and it is suggested to disperse the bacteria hence counteract aggregation (Sheikh et al. 2002). *AaiC* are located on the chromosome and it encodes an type VI secretion system (Jensen et al. 2017). The AggR is a key virulence regulator which regulates genes on the chromosome and on the pAA plasmid. *AggR* is located on the pAA-plasmid and it positively regulates AAF-genes, *aap*, *aaiC* and *aatA* (Morin et al. 2010; Nishi et al. 2003). The *aatA* is encoded on the pAA2-plasmid and it is suggested to act as a pore for Aap translocation, due to is channel-like structure (Nishi et al. 2003).

However, this method is optimized by Jesper Ingvorsen in relation to the used kit which is the Qiagen multiplex kit and the primer concentrations are adjusted (see appendix 1 for the whole protocol).

How to make primer mastermix

Primer stock is prepared by taking 10 µl of each of the 8 primer stocks (100pmol/ul) with the addition of 420 µl H_2O.

$$Primer\ stock\ 10\ \mu l\text{:}\ \frac{100\frac{pmol}{pL}}{420\ \mu l} = 42\frac{pmol}{pL}$$

The concentration of the primer stock is 42 pmol/pL.

Reaction (with Qiagen multiplex kit)

Table 2 The amount of each component for 1 reaction and for 12 reactions are shown.

	1 reaction	12 reactions
Qiagen multiplex	5 µl	60 µl
Water	2 µl	36 µl
Primer mastermix	1 µl	12 µl
		-
Template	2 ul	-

- 9 µl reaction is transferred to each PCR tube. To each tube, 2 µl DNA/template is added

Table 3 List of the genes, primer sequences and size of the fragments. The letters in parenthesis describes the location of the genes. P stands for plasmid and the C for chromosome.

Genes	**Primer sequence**	**Size (bp)**
aatA (P)	CTGGCRAAAGACTGTATCAT CAGCTAATAATGTATAGAAATCCGCTGT	**642**
aggR (P)	GCAATCAGATTAARCAGCGATACA CATTCTTGATTGCATAAGGATCTGG	**426**
aaiC (C)	TGGTGACTACTTTGATGGACATTGT GACACTCTCTTCTGGGGTAAACGA	**313**
aap (P)	GGACCCGTCCCAATGTATAA CCATTCGGTTAGAGCACGAT	**250**

PCR program

Table 4 The PCR Program with the temperature, time and number of cycles for each step

	Temperature	Time	Number of cycles
Initial denaturation	95 °C	15 min	1
Denaturation	94 °C	30 s	30
Annealing	57[a] °C	50 s	
Extension	72 °C	50 s	
Final Extension	72 °C	10 min	1
Storage	4 °C	∞	∞

Gel Electrophoreses

The gel electrophoresis is used to verify the presence of the target genes. Since the size of the expected fragments are known we load the PCR product on the agarose gel. If a band for the expected fragments are seen, we can verify the presence of the virulence genes. The four genes have a size between 250-642 bp. They vary enough in size to be distinguished on the gel. Each PCR run includes EAEC negative and positive control *E. coli* strains and PCR water to control if the PCR was performed under sterile environment. The gel was an 0,9% agarose gel and it ran 1 hour at 80V. One of the gels are shown in figure 7.

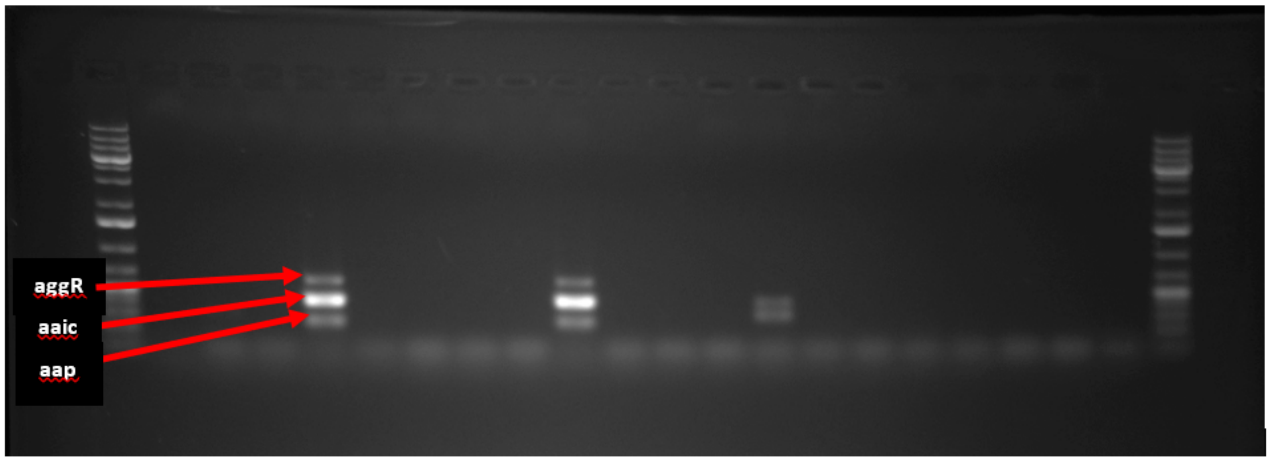

Figure 7 PCR product 121-156 loaded on the gel. 3 clear visible bands appear on the sample 124 and 129. The genes aggR, aaiC and aap is visible.

Serotyping

Serotyping is to detect and differentiate strains of microorganisms that differs in the antigenic composition. To identify different *E. coli* strains, there are tested for different surface antigens. There are identified approx. 186 different *E. coli* O-polysaccharide antigens, 53 different flagella H-antigens and approx. 80 different capsular K-antigens, but it is more complicated to test for K-antigens, therefore most serotyping for *E. coli* is based on O- and H-antigens. The conventional method of serotyping is based on agglutination reactions of O-antigens (Fratamico et al. 2016). An agglutination reaction occurs when specific antibodies cross-linking to specific antigens and forms immune complexes (figure 8). Agglutination reaction forms aggregates, that are visual for the unaided eye (figure 9) (Willey et al. 2017). Classification of EAEC by O serotyping is problematic, because many strains auto-agglutinates and there is EAEC strains that share serotypes but differs in pathotypes. They adhere differently to Hep-2 cells. There is a variety of EAEC serotypes, but the most common types according to a hospital-based study from the United Kingdom is: O126: H27, O44:H18, O111ab:H21, O73:H18, O92:H33, O126:H27, and O136:H2 (Croxen et al. 2013).

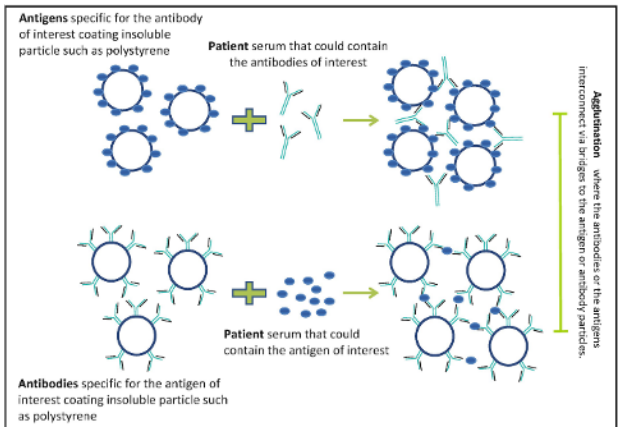

Figure 8 Agglutination reaction. Antibodies and antigens form an immune complex (Alhabbab, 2018)

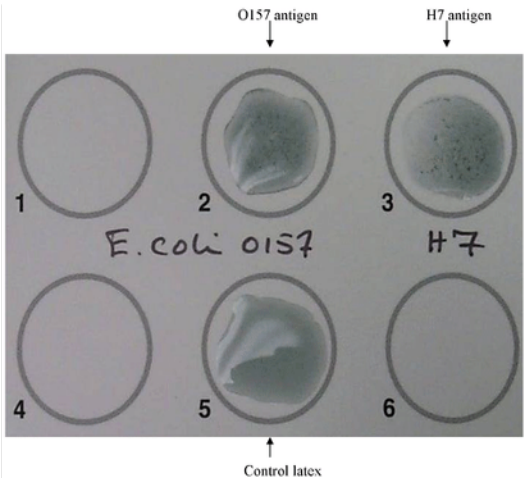

Figure 9 Agglutination reaction forms aggregates, that can be seen with the unaided eye (Peter Feng and Jinneman 2017)

Hela Cells growth and fixation

HeLa cells are the first human cell line, isolated from a cervical cancer patient. It is use by researchers all over the world because it is fast growing in cultures (Lucey, Nelson-Rees, and Hutchins 2009). The cells are grown in culture flask in growth medium at 37℃. Twice a week the cells is subdivided

into new culture flask with fresh medium, so they can continue growing (appendix 4 for cell splitting). The objective of fixation is to stop the cell growth and avoid cellular autolysis to preserve the cellular components and morphology for investigation. There are several different methods of fixation, but they can be divided into two main groups: additive and denaturing fixations. Additive fixation or cross-linking fixation forms covalent chemical bonds between proteins and in this way preserve the natural protein structures. The additive fixation solution consists of various aldehydes, including formaldehyde, paraformaldehyde and glutaraldehyde. The denaturing fixation or precipitating fixation modifies the tertiary structure of proteins and inactivates enzymatic interactions by reducing their solubility and disrupting the hydrophobic interactions. The denaturing fixation solution consist of alcohol such as methanol and ethanol, but can be used in combination with other denaturing chemicals, like acetone and acetic acid (Chao and Zhang 2011; Miqdady et al. 2002).

Phenotyping

Phenotypes is the observable characteristic of an organism. The phenotype is a result of underlying genotypes expression (Oellrich et al. 2016). Most of the EAEC strains have the characteristic stacked brick pattern which can be investigated by using adhesion assays. 3 different assays are used for analyzing the stacked brick pattern adherence to HEp-2 cells, quantification of adherent bacteria and at last a biofilm assay was performed to check the biofilm formation (Jønsson et al. 2015).

Results

The sample list with sample names are listed in the appendix 2. The pictures of the gels are in appendix 3. Some of the patients have more than one results, because of the presence of more than one *E. coli* strain. The total are 185 *E. coli* isolates from 100 patients. Table 5 Shows the number of the *E. coli* strains harboring EAEC genes both in numbers and percentage. Two diagrams for the EAEC positive *E. coli* strains are made to visualize the number of patients (figure 10) harboring *E. coli* isolates with EAEC genes (figure 11).

Table 5. The samples and the percentage of the positive samples and patients respectively

Samples:	**Positive:**
185	12 (6,5%)
Patients:	
100	9 (9%)

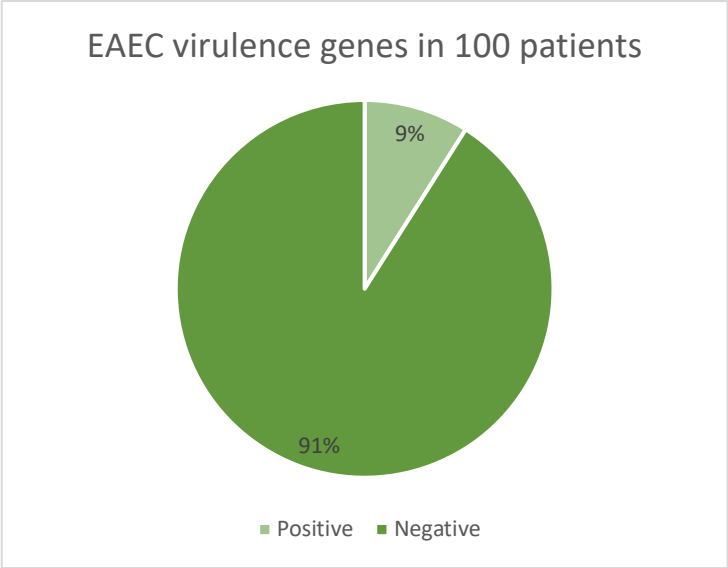

Figure 10. Diagram showing the positive and negative patients. 9% percent has at least one of the four virulence genes.

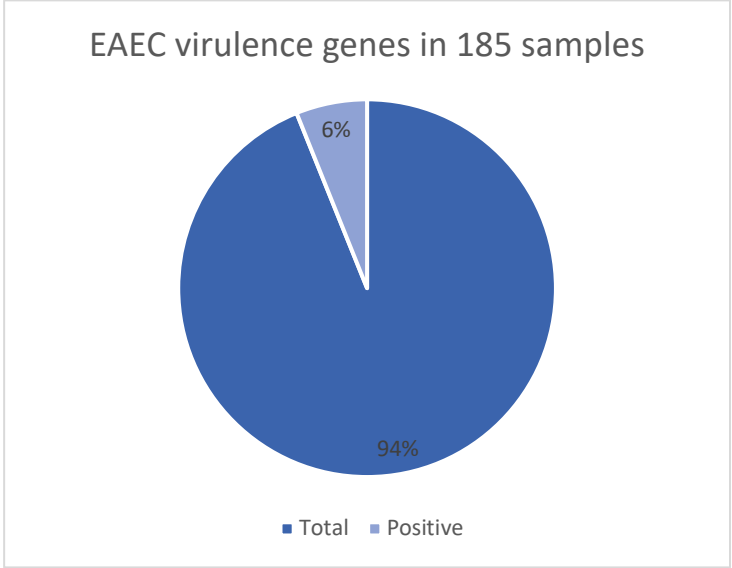

Figure 11. Diagram showing the 185 samples and approx. 6% are positive for one of the four virulence genes.

EAEC positive *E. coli* isolates are further investigated for the 4 virulence genes indicating *E. coli* phylogenetic group A, B1, B2 and D. Data for the phylogenetic groups are from the paper *"Extraintesti-*

nal pathogenic Escherichia coli are associated with intestinal inflammation in patients with ulcerative colitis." (Hengameh C. Mirsepasi-Lauridsen et al. 2016). An overview of the samples and the presence of the genes are shown below:

Table 6. A list of the positive samples and the four virulence genes listed. The samples with the virulence genes are marked with a plus sign. The control samples are listed below the positive samples. The control 042 has all four virulence genes and the other JM221 has only the chromosomal gene aaic. The EDL933 is negative for all the virulence genes and the water is also a negative control.

Sample	*aap*	*aaiC*	*AggR*	*aatA*	Phylogenetic group
33- 66068A				+	B2
48- 66148X	+				D
49- 66148A	+				D
60- 66179X	+				B2
88- 66247X	+	+	+		B1/B2
90- 66247B	+	+	+		B1/B2
91- 66247C		+			B1/B2
122- 66319X				+	D
133- 66375B		+	+		B2
124- 66331X		+	+	+	B2/D
129- 66329A		+	+	+	D
178- 66041A	+				B2
Control 042	+	+	+	+	
JM221		+			
EDL933 (negative control)					
Water (negative control)					

The distribution of the genes in the 12 *E. coli* isolates are shown in table 7 and visualized in figure 12. The distributing of the genes are close to equal and 1/3 of the *E. coli* isolates have 3 out of 4 genes.

Table 7. Percentage and the number of E. coli isolates containing each gene.

Genes	%
aap	50 (6)
aaiC	50 (6)
AggR	41,6 (5)
aatA	33,3 (4)

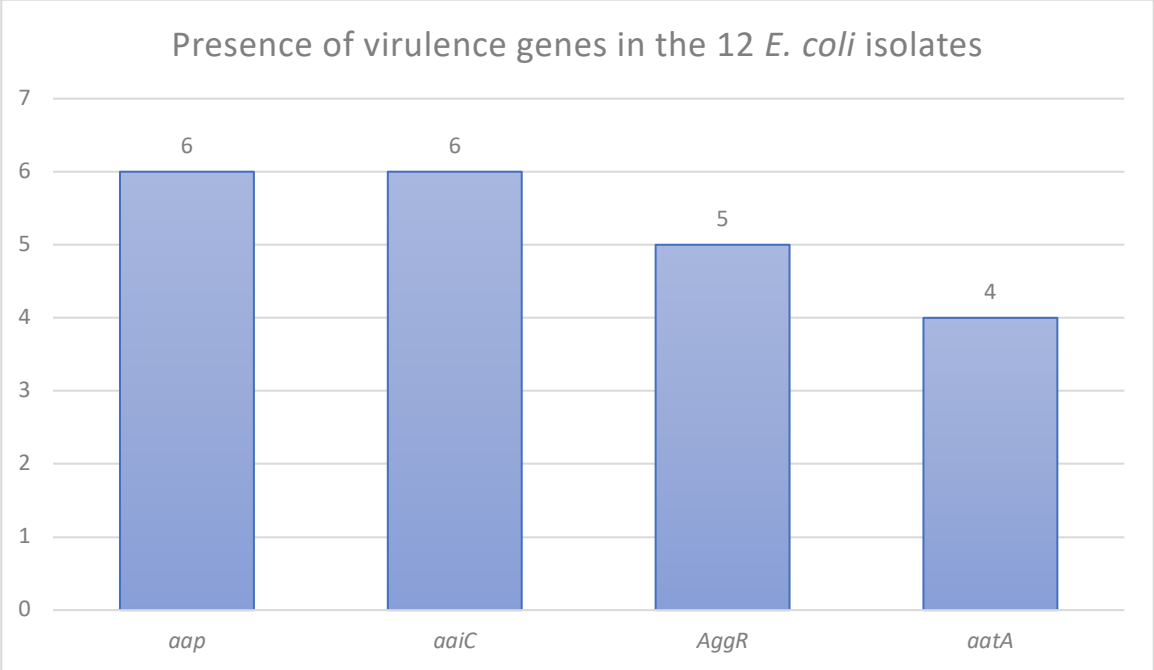

Figure 12 Graphical display of the distribution of genes in the EAEC positive E. coli isolates.

Discussion

In this section we will discuss our results from the PCR and gel electrophoresis. Furthermore, we will discuss similar studies and compare it to our results.

PCR results

Our PCR results shows that out of 185 *E. coli* isolates 12 of them had at least one clear bands in the gel-picture, which indicates presence of at least one of the EAEC genes. 9% of all the patients have at least one of the EAEC virulence genes. For further investigation each *E. coli* isolates harboring EAEC genes, were analyzed by PCR again and PCR product was run on the agarose gel. 41,6% of the positive samples had the AggR-regulator which is expected since the AggR-regulator is crucial for the regulation of the virulence factors (Sheikh et al. 2002). EAEC without the AggR regulator are categorized as an atypical EAEC and strains with AggR are suggested to be more virulent (Sarantuya et al. 2004). A mice study shows that an AggR mutant lacking the AggR regulator, had reduced morbidity and mortality in Shiga toxin producing (stx) EAEC (Boisen et al. 2019). Hence, the AggR-regulon is proposed to have a major impact in the virulence and pathogenesis of the EAEC, and the lack of AggR likely lowers the *E. coli* virulence isolated from UC-patients with active disease. The virulence of the atypical EAEC cannot be excluded. The atypical strains can be as virulent as typical strains which is shown *in vivo* in the *Galleria Mellonella* (wax moth) model (Guerrieri et al. 2019).

The dispersin protein encoded in *aap* is found in approx. 50% of the samples and most of the samples with dispersin do not contain other virulence factors (see table 6 and 7). The virulence of the samples with only dispersin and without the AggR-regulator is doubtful since the dispersin are positively regulated by AggR. However, it cannot be eliminated since the epithelial barrier are disrupted in UC patients with active disease, hence an enhanced uptake of antigens are possible (Gitter et al. 2001).

Furthermore, the samples containing *aap* does not encode *aaiC*, which is the only gene located on the chromosome hence the dispersin gene could be acquired through horizontal gene transfer and it could be doubtful whether these bacteria are EAEC or another *E. coli* since the phenotyping and

serotyping is not performed. The presence of *aap* is found in both EAEC, DAEC and other nonpathogenic strains, which also supports the hypothesis that the samples with only *aap* is probably not EAEC (Monteiro et al. 2009). Based on these results and the *E. coli* isolates with 3 EAEC virulence genes including *aaiC* can be presumed to be virulent EAEC. The samples with only one virulence genes could be incorrect and further analysis were not possible because of the covid-19 situation. Interestingly the PCR results from our study is similar to a study conducted in Guatemala and Mexico (Bamidele, Jiang, and Dupont 2019). The samples with AggR also had the *aaiC* gene, like our 5 samples with aggR + *aaiC*. But this study also lacks the phenotyping where the stacked brick pattern normally is confirmed.

The phylogenetic groups of the positive samples are mainly B2 and D. The B2 and D are mainly virulent extra-intestinal strains (Saralaya et al. 2015). From the obtained results it could be suggested that the samples have ExPEC properties.

Other studies

In the study *"Phylogenetic and pathotype analysis of Escherichia coli stool isolates from Egyptian patients with inflammatory bowel disease"* the content of bacteria in Egyptian patients with IBD is investigated (Meheissen, Header, and Abdelaty 2019) . 80 patients are included in the study where 30 patients are diagnosed with CD and 30 with UC. 20 individuals are control subjects. Several multiplex PCR are performed to detect virulence genes for EAEC, EHEC, EIEC, EPEC, ETEC and STEC. All the genes and the sequence for each pathotype is listed in the article. For the detection of EAEC the primers consist of sequences from the pAA plasmid (CVD 432). The CVD 432 encodes the *aatA* gene (Monteiro et al. 2009).

The results from the paper is shown below:

Table 8 displays the study groups and the percentage of the patients with the different E. coli pathotypes and which phylogenetic groups they belong (Meheissen, Header, and Abdelaty 2019).

	Active UC (n = 15)	Inactive UC (n = 15)	Active CD (n = 15)	Inactive CD (n = 15)	Controls (n = 20)
E. coli pathotype					
EAEC	3[a] (20%)	3[a] (20%)	6[a] (40%)	3[a] (20%)	0[b] (0%)
ETEC	0 (0%)	1 (6.7)	0 (0%)	1 (6.7%)	0 (0%)
EPEC (atypical)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (5%)
E. coli phylogenetic group					
A	6 (40%)	4 (26.7%)	5 (33.3%)	6 (40%)	10 (50%)
B1	0 (0%)	2 (13.3%)	0 (0%)	0 (0%)	0 (0%)
B2	6 (40%)	7 (46.7%)	8 (53.3%)	9 (60%)	10 (50%)
D	3 (20%)	2 (13.3%)	2 (13.3%)	0 (0%)	0 (0%)

The results show the presence of EAEC in all the study groups except for the controls. 15 out of 60 samples had EAEC which gives 25% of all the samples. The highest amount is found in the active CD patients with 40%. Other DEC's are almost not present in any of the study groups. The phylogenetics groups are also identified and 80% of the EAEC were from group B2 and D, where the rest were assigned to group A.

The disadvantages for (Meheissen, Header, and Abdelaty 2019) are the selection of genes for detection of EAEC. The CVD 432 is part of the plasmid and bacteria can obtain plasmids from other bacteria due to the horizontal gene transfer mechanism. Hence a better virulence gene to detect would be a gene located on the chromosome of the bacteria. From the list of virulence genes in EAEC it is clear that most of the genes are located on the plasmid and this could be the reason that the plasmid is detected rather than the only two genes on the chromosome (table 1). The EAEC strains are very heterogenous and the classification can vary. Some classify the bacteria as EAEC if they make the stacked brick pattern and have the AggR regulator while others also detect for the presence of some other virulence genes (Aslani et al. 2011; Estrada-Garcia and Navarro-Garcia 2012). Compared to our results the prevalence of EAEC is higher in the (Meheissen, Header, and

Abdelaty 2019), this could be explained with geographic factors, like sanitary and dissimilarities between Danish and Egyptian patients.

In the paper *"Extraintestinal pathogenic Escherichia coli are associated with intestinal inflammation in patients with ulcerative colitis."* (Hengameh C. Mirsepasi-Lauridsen et al. 2016) a study is conducted where the influence of the *E. coli Nissle* (EcN) is used as add-on treatment to conventional therapies for active UC patients. The study is based on the knowledge that stool samples from UC patients has an increased number of *E. coli* with ExPEC genes. EcN also belongs to the B2 phylogenetic group. The results of the experiment showed that the treatment with EcN did not promote remission in active UC patients with active disease, on the contrary it increased intestinal inflammation. IBD patients have a high prevalence of *E. coli* belonging to the B2 group which might be because of their metabolic capabilities. It is suggested that B2 strains has distinct metabolic properties that make them capable of utilizing energy more efficiently (Fang et al. 2018). This could explain the increased intestinal inflammation in patients treated with EcN.

The results in this study and the study (Meheissen, Header, and Abdelaty 2019) indicates that the EAEC found in the Egyptian UC and CD patients also could be a contributor to the intestinal inflammation. The EAEC found in the (Meheissen, Header, and Abdelaty 2019) study also belongs to the B2 phylogenetic group and the acquiring of ExPEC genes in DEC could lead to a not well defined pathogenetic group, since the patients has diarrhea but the detected *E. coli* are more linked to ExPEC (Patzi-Vargas et al. 2015).

AIEC

Another interesting DEC group which is associated with CD are the AIEC. AIEC which is associated with CD could be a combination of an ExPEC with invasive properties as the DEC group. CD-patients has *E. coli* with DEC pathotypes which is combining several virulence factors (Da Silva Santos et al. 2015). Studies show that AIEC also share both genetically and phenotypically features as ExPEC (Martinez-Medina et al. 2009). Identification of AIEC are challenging since it is very heterogenous, and they are identified by testing their ability to survive and replicate in macrophages (O'Brien et al. 2017; Robins-Browne et al. 2016). In a study conducted on Korean IBD patients, the AIEC are

found in CD- and UC-patients at similar rates. It is suggested that AIEC are associated with sustaining mucosal inflammation. 44,2 % of the *E. coli* found in the patients, belongs to the B2 phylogroup (Lee et al. 2019). These results indicates that the AIEC found in Korean IBD patients (Lee et al. 2019) also harbors ExPEC properties as in the (Martinez-Medina et al. 2009).

DAEC

DAEC is linked to UC and genes linked to DAEC could also be present in the *E. coli* isolates from UC patients, but it remains challenging to detect since it is very heterogenous (Lopes et al. 2005). Moreover, this pathotype is found in children with diarrhea (Girón et al. 1991; Knutton et al. 2001; Levine et al. 1993). The relation between pediatric IBD are investigated and DAEC are found both in UC- and CD-patients. DAEC is suggested to be considered as an pathobiont in pediatric IBD (Walczuk et al. 2019). The literature lacks data where the DAEC are investigated in relation to adults with IBD, hence the association between DAEC and adult IBD-patients remain weak.

Treatment

Patients with IBD has a higher *E. coli* prevalence and it is suggested to impact the inflammation in the colon. Therefore, studies investigating the effect of antibiotics are performed. The results are varying in each study. Treatment with antibiotics in Danish patients with diarrheagenic EAEC did not reduce the duration of diarrhea. The EAEC strains had a high antibiotic resistance which could explain why the antibiotics did not have any effect (Hebbelstrup Jensen et al. 2018). It is important to notice that this study has not included IBD patients hence in practice the results can not directly be interpreted. Treatment with antibiotic in IBD management are suggested but the effects of the antibiotic are still unclear. The effect could be due to the alteration in one bacterial species or treatment of another secondary infection (Ledder 2019). Another important factor could be that a lot of bacteria acquire multidrug resistance hence the antibiotic would have no effect.

The immunosuppressive are the best treatment for IBD patient with active disease yet, but it has also side effects when used on the long term (O'connor, Qasim, and O'morain 2010). Immunosuppressive treatment also increases the risk of opportunistic infections such as *clostridium difficile* and *E. coli* , which are the mostly frequent (Axelrad et al. 2017; Gong et al. 2019). As a conclusion the

treatment of IBD remains still challenging and more research is needed on key bacteria linked to IBD and specific antibiotics as a treatment against that specific bacteria/ *E. coli*.

Conclusion

Based on our results we can conclude that the presence of EAEC are associated with UC. The multiplex PCR results showed that 9% of the patients have one or more of the *aap, aaiC,* AggR*, aatA* virulence genes found in EAEC. The *E. coli* isolates harboring AggR + *aaiC* (41,6%) genes are assumed to be EAEC since the crucial regulator AggR and the chromosomal gene *aaiC* are present. Moreover, the phylogroups of the EAEC positive *E. coli* isolates belonged to B2 and D phylogenetic group, which confirmed by other studies and it is high likely that the found EAEC positive *E. coli* strains also has ExPEC properties. The results indicate that the EAEC has an importance in the research of the pathogenesis of UC. Furthermore, since the samples are not further analyzed with serotyping and phenotyping some of the *E. coli* isolates with only one gene present is doubtful EAEC. The results show *E. coli* with virulence properties hence treatment with antibiotics could be beneficial but further studies are needed.

Perspectivation

Further work/ investigation with focus on UC disease activity related to EAEC could be relevant. This could be performed by analyzing the EAEC positive *E. coli* isolates, where the *E. coli* isolates were phenotyped to confirm if the EAEC positive strains make the characteristic stacked brick pattern. Furthermore, the presence of AAF could be investigated in the *E. coli* isolates, which adhere to Hela-cells. The other negative-*E. coli* isolates could be analyzed and categorized under the other *E. coli* strains/types such as DEC. We believe the most relevant groups to investigate in UC patients are DAEC since it is linked to UC and the AIEC, in CD, since it is linked to CD. This could lead to a better classification of increased prevalence of the *E. coli* in IBD patients with active disease, which will lead to better /antibiotic treatment in IBD to promote disease remission. The DAEC is primarily investigated in pediatric IBD patients hence investigating adult IBD-patients could explain if there is any association in adult IBD.

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