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Cancer and Infection

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Cancer and Infection

by

Kathleen H. Plummer

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science
Department of Cell Biology, Microbiology, and Molecular Biology
College of Arts and Sciences
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DEDICATION

To my beloved grandparents, thank you.

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ABSTRACT

E. coli is the most frequently isolated Gram negative pathogen from bacteremia in cancer patients and is repeatedly recovered from many other extraintestinal illnesses. These infections are commonly endogenous in nature and interfere with the treatment of cancer resulting in increased healthcare costs, morbidity, and mortality rates. Cancer and the treatments related to cancer cause alterations in the microbiome of the gut and other organs. Despite this point, there is a serious lack of knowledge about the genetic types of *E. coli* infecting cancer patients. This gap results in vague prevention strategies and limited treatment options for cancer patients. Multi Locus Sequence Typing (MLST) was used to successfully genotype 105 sequentially collected *E. coli* isolates from patients admitted to H. Lee Moffitt Cancer Center (HLMCC, Tampa, FL) with confirmed extraintestinal infections between 2010 and 2012. In total, 24 distinct genotypes (STs) have been identified in this dataset using *Ec*MLST (STEC Reference Center). Of these, ST34 constituted 39% of the sample and may represent a disseminating clone at HLMCC. Furthermore, 17 isolates not found in the *Ec*MLST database have been identified. Importantly, phylogenetic analysis of DNA sequence data for HLMCC *E. coli* revealed only 22% of HLMCC *E. coli* clustered with ECOR reference strains commonly attributed to the B2 phylogroup of extraintestinal pathogenic *E. coli* (ExPEC). Four HLMCC *E. coli* belonging to ST171 and attributed to life-threatening blood infections clustered with Shiga toxin (Stx) producing *E. coli* (STEC) strain TW06296. HLMCC *E. coli* belonging to ST34 clustered with enteroaggregative *E. coli* (EAEC) strain TW10263. Importantly, these non-B2 phylogroup strains demonstrated more pathogenic potential than HLMCC *E. coli* clustered with B2 ExPEC,

which included a higher incidence of bacteremia and sepsis, as well as resistance to first-line antibiotics. Upon further investigation, ST34 may equate to ST131 by another MLST database. These findings suggest that isolates previously characterized as commensal and intestinal pathogenic *E. coli* have an increased ability to cause infection outside of the gastrointestinal tract in cancer patients and that selective pressures are contributing to increased antibiotic resistance. These findings may change the approach to clinical management of *E. coli* infections at cancer centers.

CHAPTER ONE:

CANCER AND INFECTION: CONSEQUENCES OF THE IMMUNOCOMPROMISED IMMUNE SYSTEM AND SUSCEPTIBILITY TO INFECTION

Cancer- Overview

Cancer is an expansive term for many diseases in which abnormal cells uncontrollably divide and spread. As a leading cause of morbidity and mortality, it warrants serious investigation and intervention of global concern. In 2012, cancer plagued 14 million people worldwide. Furthermore, over 8 million deaths were attributed to cancer and more than 32 million people diagnosed in the past 5 years are living with the cancer diagnosis (5 year prevalence) (1, 2).

In healthy organisms, abnormal cells are controlled by induction of apoptosis through tumor suppressor genes. In cancer, mutations in these and other genes results in repression of apoptosis and subsequent proliferation of atypical cells. Rapidly dividing cancer cells initially cause tumors in a primary tissue source. Then cells can invade nearby tissue resulting in cancer of surrounding areas. Additionally, invasion into the circulatory system is the vehicle by which malignant cells metastasize. Metastasis catapults malignant cells through the circulatory system which allows for invasion of multiple distant tissues (3).

Although incidence rates vary among cancer type and geographical region, the average rate for men in 2012 was 205 per 100,000 ranging from as low as 79 per 100,000 in Western Africa, to

as high as 365 per 100,000 males in the region of Australia and New Zealand, due to significant prostate cancer diagnoses. Women tend to have lower incidence rates, averaging 165 per 100,000 and ranging from 103 per 100,000 in South-Central Asia to 295 per 100,000 women in Northern America (1). Unfortunately, many of these diagnoses occur in considerably less developed regions where the burden of cancer and treatment is even more agonizing. It was estimated that nearly 8 million new cases, 5.3 million deaths and 15.6 million patients with 5 year prevalence diagnoses occurred in less developed regions in 2012. These regions have decreased access to prevention, diagnosis and treatment (4). Non-fatal health outcomes are another concern when considering the overall effects of cancer on a community. In 2010 an estimated 7.6% of global burden of disease was attributed to neoplasms (5).

Regrettably, the causes of cancer are just as innumerable as the cancers themselves. Although instances of cancer have been documented as far back as ancient history, the multiple origins of cancer are still under investigation. Analysis of cancer biology and genetics has allowed us to understand specific processes, like DNA repair deficits, errors in DNA replication and alterations in chromosomes, which result in malignancy. However, the influencers that lead to these problems are largely unspecific. A few known stimuli include genetic chromosomal mutations, inflammation, radiation, chemicals and viruses (6).

This broad generalization of cancer, which impacts the lives of millions of people, is well studied. According to the National Cancer Institute, the United States spends an average of \$4.9 billion in research each year. Although great strides have been made in the area of cancer research, its variability is still perplexing. Midst the many fields of exploration, advances in the differentiation of malignant cells have helped to determine type, treatment, and response. Cancer can be categorized according to the location or cell type from where the proliferation of

malignant cells originated. According to the National Cancer Institute, there are over one hundred different types of cancer. However, in most instances all of these can be assembled into five major categories which include: carcinoma, leukemia, lymphoma and myeloma, sarcoma, and central nervous system cancers (3).

Carcinoma

Carcinoma makes up the overwhelming majority of illness, surgery, and death by cancer worldwide. Carcinoma originates in one of the many types of epithelial cells. The most common cell types seen in this disease include glandular, basal cells, squamous cells, and transitional cells (3). Basal epithelial cells line the outermost layer of the epidermis. As such, they are most frequently found on body parts likely damaged by exposure to UV sunlight and are therefore the most abundant form of skin cancer (3). Squamous cell carcinomas (SCC) also account for approximately 700,000 cases of skin cancer in the United States annually (7). Together, these Non-Melanoma Skin Cancers (NMSCs) are the leading cause of cancer in the United States and they are increasing. Between 1992 and 2006 NMSCs treatments increased by a striking 77 percent and they account for an estimated 3.5 million diagnoses every year in the United States (8). Approximately 90% of NMSC diagnoses are associated with UV sunlight radiation (9). Additionally, recent increases in indoor tanning have played a role in the increase of NMSCs. An outsized analysis of non-melanoma skin cancer, comparing use of indoor tanning and the absence of indoor tanning, calculated a relative risk for SCC and BCC of 1.67 and 1.29 respectively, alluding to a contribution of 170,000 cases of NMSC by indoor tanning each year (10).

Squamous cell carcinoma can invade considerably more than the superficial layers of the skin. Squamous cells also line the respiratory tract, digestive tract, and hollow organs. Squamous cell carcinomas include cancers that originate in the anus, cervix, head and neck, and vagina. Transitional cell carcinoma presents in similar sites, as transitional cells line the hollow organs of the body as well (3). According to 2012 global estimations, with the exclusion of non-melanoma skin cancer, cervical cancer was the fifth leading cause of cancer in women and accounted for 7.5% of cancer deaths in the female population (1). The majority of cervical cancer cases are associated with the Human Papillomavirus (HPV) (11). Furthermore, women infected with HPV who smoke or are exposed to smoke have an even greater risk of developing cervical cancer (12). Contrarily, cervical cancer in the United States is much lower with 12,340 (0.7%) newly diagnosed cases and just over 4 thousand deaths. This decline is attributed to regular Papanicolaou and HPV screenings as well as the HPV vaccine (13, 14).

Glandular cells are designed to secrete fluids like mucus, digestive enzymes, and milk. Adenocarcinoma is the resulting cancer from mutations and proliferation of these cells which accounts for most breast, pancreas, lung, prostate and colon cancers(3). In 2012, lung cancer alone accounted for an estimated 13% of new cancer diagnoses, a notable 19.4% of cancer mortalities and 5.8% of patients living with a five year prevalence (1, 2). Lung cancer claimed over a million lives, making it the leading cause of cancer mortality (1). This substantial death rate is attributed to smoking, as there are more than 60 carcinogens found in cigarette smoke. These carcinogens alter the tissue barrier that protects the lungs from multiple environmental challenges thus resulting in increased bronchial epithelial permeability (15). The risk ratio for lung cancer is greater than 6 when comparing smokers to non-smokers (16). This is of no surprise considering mutagens in tobacco smoke modify DNA. Studies suggest that

transcription-coupled repair pathways as well as expression-linked repair pathways are changed in lung cancer cells (17).

Breast cancer held the second highest estimated incidence rating of 11.9% with a mortality rate of 6.4% and a five year prevalence of 19.2% (1, 2). Breast cancer is frequently seen when mutations have occurred in specific tumor suppressor genes. For example, women with inherited mutations in BRACA1 and BRACA2 and to a lesser extent, PTEN and TP53, are at a much greater risk for developing breast cancer (18). These mutations are not the cause of cancer itself but they are indicative of the patient inability to defend against malignancy in glandular carcinomas. Rather, extensive research in various mutations seems to suggest that multiple acquired mutations are to blame for most breast cancers (19-21). These mutations can be caused by environmental factors like radiation, carcinogen exposure, and other unknown risk factors. Detection of specific acquired mutations, like multiple copies of HER2, may help to determine the aggressiveness of treatment therapy and predict patient outcomes to various types of chemotherapeutic drugs (22-24).

Prostate cancer is the second leading cancer diagnosis for men worldwide. Prostate cancer accounted for 7.9% of all cancer incidence rates in 2012 and 3.7% of cancer mortalities. Furthermore, currently over three million men have a 5 year prevalence of prostate cancer. However, the frequency of prostate cancer between racial backgrounds is divergent. In Western countries, this is the leading cancer diagnosis for men (1, 2). The highest rates are among African American men in the United States. This population has a 60% higher incidence rate than Caucasians with 116 per 100,000 men being diagnosed. Chinese men on the other hand have a prevalence of only 28 per 100,000 (25). The varying severity of prostate cancer is also a conundrum. Many cases remain asymptomatic while others result in death. Epidemiological

studies support an inherited component to increased risk for prostate cancer. Familial clustering associations were much stronger than age or race matched control groups. Additionally these heredity patterns appear more robust than the heredity patterns seen in breast and colon cancers (26). Other reasons for the differences in potency may include therapy, slow tumor growth or progression before diagnosis (27). Genome-wide studies suggest both numerous variants and common patterns implying both genetic and environment factors (28).

In 2012, colorectal cancer (CRC) accounted for 9.7% of new cancer cases, 8.5% of cancer mortalities and 10.9% of patients with a 5 year prevalence rating. Rates are consistently higher among men than women and in western countries but show no significant differences between more or less developed regions (1, 2). Although, the majority of colorectal cancer cases are considered random, studies suggest that an individual or familial history of non-neoplastic polyps may increase risk (29-31). This may be an innate or acquired tendency for tumor formation in the colon which may or may not undergo malignant transformation. Comparisons of genetic alterations in normal and malignant colon epithelium suggest that the transformation is a result of multiple acquired molecular events that result in chromosomal instability (32-34). Sequencing of CRC genes established an average of 90 mutant genes. 69 of those were considered relevant to pathogenesis of colorectal cancer and an average of nine mutant genes were seen per tumor (35). A plethora of mutations in DNA damage repair genes and the DNA mismatch repair system allow for more rapid mutations in cancer-associated genes (36).

These four leading glandular cancers account for over 38% of the estimated cancer deaths in 2012. Although stomach and liver cancer do not retain as high of an incidence rate, their high mortality rates of 8.8% and 9.1% respectively also contribute greatly to the burden of mortality by carcinoma (1). Altogether the above cancers account for almost 56% of all cancer mortalities

excluding non-melanoma skin cancer in 2012. In other words, they were the cause of over 4.5 million deaths (1).

Leukemia

Leukemia is hematological cancer that starts in the blood-forming tissue, bone marrow, and spreads through the body by way of the circulatory system. There were over 350,000 new diagnoses of leukemia in 2013 and over 265,000 deaths. These hematological cancers are differentiated by rapacity of onset and the cell line from which they originated. This includes either myeloid or lymphoid stem cell lines and these are further delineated by acute or chronic onset of disease. Men have higher incidence than women and Caucasians have higher rates than any other ethnic group (3). The treatment of leukemia generally requires induction therapy which results in prolonged neutropenia and a high risk of infection.

Leukemia is the most common cancer in children, acute lymphoid leukemia (ALL) being the most abundant. Innovations in treatment have drastically impacted childhood cure rates which are now greater than 80% (37). However, ALL accounts for 20% of all acute leukemia in patients over 20 whose long-term disease free survival is only 30-40% (38). This is a result of the high frequency of diverse genetic abnormalities in cancer cells, a greater severity of toxicity from chemotherapy and higher incidence of comorbidities (39). Infections remain the main causes of morbidity and mortality in ALL patients due to induction chemotherapy and reduced bactericidal activity of neutrophils (40, 41). While innovation in treatments allows for a decrease in mortality rates, increases in diagnosis are seen each year (39).

Various factors play a role in each of the different types of leukemia. Chronic myelogenous leukemia (CML) patients possess a DNA translocation called the Philadelphia chromosome

which results in excess enzymatic production of tyrosine kinase resulting in undue production of incompetent white blood cells. This same translocation is occasionally seen in acute myelogenous leukemia (AML) and acute lymphocytic leukemia (ALL) (3). Various other gene fusions, chromosomal rearrangements, and translocations are contributing factors to these hematological cancers (42, 43).

Lymphoma and Myeloma

Lymphomas are neoplastic diseases that originate in the lymphatic system. Lymphomas are a diverse group of diseases as they are the fifth most common group of cancers in the United States. Lymphomas are divided into Hodgkin's and non-Hodgkin's based on distinguishing cell characteristics. Hodgkin's lymphoma (HL) presents in the lymph nodes and possesses Reed-Sternberg cells. Hodgkin's lymphoma is a result of acquired DNA damage to lymphocytes and is currently one of the most curable cancer types. Non-Hodgkin's lymphoma (NHL) is much more diverse and can vary by aggressive or indolent growth. According to the WHO there are over 60 sub-types of NHL. Due to these dissimilarities, treatment and survival rates for NHL are inconsistent. In 2012, incidence of Hodgkin's lymphoma were almost 66,000 while non-Hodgkin's accounted for almost 386,000 globally (1, 44-46).

Myeloma is another hematological cancer which is commonly referred to as multiple myeloma (MM). MM affects lymphocytes of B cell lineage resulting in over proliferation of malignant plasma cells. Plasma cells are responsible for immune protection via antibody production. Furthermore, they maintain integrity of bones by production of osteoclast activating factor. Myeloma cells then cluster in the bone marrow and cause a cascade of immune breakdown. Symptoms of the disease include bone lesions due to rapid growth of myeloma cells,

hyperkalemia and renal failure due to abnormal proteins, M proteins, deposited for filtration. Abnormal proteins found in the urine known as Bence Jones protein are another manifestation of MM. Anemia is commonly seen in MM as well resulting from a decrease in normal bone marrow cell production. These patients have increased risk for infection because they are lacking in functional antibodies. The median survival is between 3 and 4 years. MM is twice as common in African Americans in comparison to European-Americans and is also more common in men (47, 48).

Sarcoma

Sarcomas are of mesodermal origin which includes connective or supportive tissues. These tissues include bone, cartilage, fat, striated muscle, and blood vessels. When malignancy is exclusive to bone it is termed osteosarcoma. Malignancy in bone and soft tissue is Ewing sarcoma. Although sarcomas are considerably rare, they predominately affect children and adolescents. Estimations suggest that less than 15,000 Americans will develop this form of cancer each year. There is currently no link to heredity of this disease and most instances can be attributed to a translocation of chromosomes. Ironically, the only other known risk factor for the disease is radiation therapy which is used to treat certain cancers

Central Nervous System Cancers

Central nervous system cancers predominantly begin sporadically in the tissues of the brain and spinal cord (CNS). An estimated 256,000 new cases occur globally, 24,500 of those being in the United States. Brain tumors are the leading cause of death from CNS cancers and make up 27% of childhood cancers. An estimated 3,200 children will be diagnosed with this cancer in 2013. Men are at a higher risk for acquisition and mortality from CNS cancers and Caucasians are

more affected than any other racial group (49). Robust associations exist between brain cancer and specific inherited syndromes in which tumors are frequent, such as neurofibromatosis type 1 and 2, tuberous sclerosis, von Hippel Lindau disease and Li Fraumeni, Gorlin and Turcot syndromes (50). Secondly, exposure to carcinogenic compounds found in occupational environments may play a role in CNS and brain cancers (51, 52).

Clearly the abundance of cancers and the multifactorial origins of cancer are more complex than we have managed to cover in this text or in the current medical community. Furthermore, the secondary effects on patients treated for these terrible diseases raise ongoing questions regarding the impact of current treatments on the burden of disease and the severity of treatment related ailments.

Compromised Host Defense- Cancer and Therapy

The human body has a multitude of protective mechanisms. A healthy individual employs mostly nonspecific resistance to pathogens continuously through intact skin and mucous membranes, excretory functions, cilia, microflora, naturally secreted antibodies, complement, lysozymes and genetic factors. These initial responses serve to delay or destroy foreign pathogen invasion while activating specific immune responses. Interruption or overstimulation of this general reaction results in a targeted response by the immune system (53). The specificity of this adaptive response varies but it can include white blood cells such as lymphocytes, phagocytes, and natural-killer cells (54). In cancer patients, immunodeficiency is a common problem resulting from the disease itself, chemotherapy, and/or radiation therapy. A severe reduction in the absolute number of neutrophils below normal range, called neutropenia, is a

frequent result of intensive chemotherapy and radiation. In turn, the body's immunity resistance is jeopardized to both native organisms and foreign pathogens.

Treatment of cancer often requires impairment of many other natural immune responses. One major contributing factor to this impairment is breakdown of the integumentary system, the skin barrier. When kept intact, skin is one of the most important means of infection prevention as it is impenetrable and even damaging to most harmful pathogens. Any trauma or puncture to the skin is a risk factor for infection by both endogenous and exogenous microorganisms. Unfortunately, the treatment of cancer requires disturbance of the skin; the most obvious example being the removal of a malignant tumor. Cancer patients may also require intravenous medicines, fluids and nutrition on a continual basis. This medical necessity leaves the patient with a constant penetration of the skin by a catheter or other indwelling medical device. Invasive medical devices like endotracheal intubation and mechanical ventilation, are also associated with poor prognosis in ICU cancer patients to the extent that cancer centers have attempted to use noninvasive continuous airway pressure in order to avoid the imminent repercussions of severe pneumonia (55). Even standard monitoring of patient status frequently requires blood draw which may result in hematoma (56).

Furthermore, skin toxicity, which can present in the forms of rashes, dryness, pruritus, paronychia, and even hair abnormalities, is a repeatedly documented side effect in cancer therapeutics (57). For example, solid tumor cancers can be treated with epidermal growth factor receptor tyrosine kinase inhibitors. Preventing the activation of tyrosine kinase receptors averts potential increases in tumor cell proliferation and the concurrent affects. Treatment with these drugs in specific cancers, like metastatic non-small cell lung, seems to fare better than more

common chemotherapy regimens (58). However, adverse effects of skin toxicity result in more than 80% of patients (59).

The skin is often the first organ affected in graft-versus-host disease (GVHD). GVHD is an immune response of donor T-cells attacking host cells post bone marrow transplant or stem cell transplant (60). These visible disruptions of skin from immunological and inflammatory responses leave the body vulnerable to infection. Unseen changes resulting from immune responses include alterations in pH, changes in structure and the ability of the skin to shed. This modifies the environment of the skin and subsequently changes the colonizers of the skin. Alterations in the integumentary microbiome can cause nosocomial infections from both endogenous and exogenous pathogens (61).

Treatment regimens of chemotherapy and radiation can also disrupt the protective components of mucosal membranes. Mucositis can occur in the mouth and the GI tract. These cells, like cancer cells, are rapidly dividing. Consequently, targeted chemotherapy or close proximity radiation treatment may damage both cancer cells and endometrial cells of the mucosal membrane. This damage makes normal tissue repair of damaged DNA and cell division challenging.

Normally, secretions from mucous membranes protect the body from invasion of microorganisms through multiple mechanisms. Many secretions contain antimicrobial properties. When IgA is present it prevents pathogen attachment to host cell receptors and causes agglutination of some microorganisms. Additionally, secretions contain iron binding proteins that compete with microorganisms for iron (53). The breakdown of these protective mechanisms results in an imbalance of microorganisms and an exposed surface making invasion

of both asymptomatic colonizers and pathogenic bacteria, as well as reactivation of viruses, an easy feat (62).

Moreover, damage to the fibronectin barrier that protects the epithelial cells is a result of ageing and illness, like the cancer itself. This factor may allow for unsuitable colonization of the oropharynx with gram-negative bacteria (GNB) instead of the normal gram-positive bacteria. Colonization then increases the likelihood of pneumonia of GNB origin in cancer patients. One example indicated a strong correlation, as 23% of colonized GNB intensive care patients presented with GNB pneumonia while only 3% of non-colonized patients had a GNB pneumonia infection (63-67).

Normal flora of the gastrointestinal (GI) tract protects against colonization from aerobic exogenous microbes. This environment contains over 400 obligate anaerobic species. In addition to protection from external organisms, endogenous anaerobes also prevent overgrowth of common GNB in the GI tract. Antibiotics used to protect neutropenic cancer patients may disrupt this cantankerous balance which can be deadly, especially when mucous membranes are disturbed by cancer treatment. Frequent antibiotic therapy for patients undergoing cancer treatment can result in destruction of anaerobic bacteria in the gut thus halting the competitive environment and sanctioning excessive growth of GNB. Once overrun, GNB can then attach and invade the compromised walls of the GI tract resulting in bacteremia. However, treatments with antibiotics that do not fight against anaerobic bacteria increase risk of infections like enterocolitis by overgrowth of anaerobes. The GI tract is notably the most important endogenous source of GNB found in the body (68-71).

Reversely, the urinary tract is a sterile system frequently afflicted by GNB. It is well known that urinary tract infections are one of the most commonly encountered problems in healthcare. The immunocompromised immune system, by way of cancer and its treatment, can work in congruency with catheterization and instrumentation to promote urinary tract infections in cancer patients even more so. Catheterization can be necessary for cancer patients. Some drugs require irrigation in order to prevent damage to the bladder. A lack of host defenses like functional phagocytes and secretory IgG and IgA, along with impeded free flow of urine, adequate bladder emptying and intact epithelial lining creates a hatchery for entry and proliferation of microorganisms up the ureters and into the bladder. Furthermore, damage to bladder mucosa by chemotherapy or instrumentation can also predispose to infections. Multiple paraneoplastic syndromes can cause urine stasis by incomplete bladder emptying. Tumors that originate in or close to the urinary tract, prostatic enlargement, or prostate cancer, can cause an obstructed flow of urine which may block part of the organ from emptying and allow for overgrowth of microorganisms. A hyperuricemic state created by hyperkalemia, tumors or the treatment of tumors may also cause stones in the bladder and obstruct urine flow. Conditions or tumors involving the spinal cord that cause neurogenic bladder can cause incomplete bladder emptying and result in infection as well (72).

Currently the most beneficial cancer treatments, like radiation and chemotherapy, are also the origins by which many patients develop life-threatening infections. Furthermore, use of broad-spectrum antibiotics on immunocompromised cancer patients results in selection of resistant pathogens for infection which in turn results in high mortality. Weakened immunity including neutropenia, innate immune disruption and altered microflora consequently allow for infections of bacterial, fungal and viral origin.

Infection-Overview

Infections are to blame for approximately half of the deaths in cancer patients (73). An immunocompromised patient is susceptible to infection because they are lacking in basic immune functions. Furthermore, chemotherapy related neutropenia is a serious cause of this immune deficit in cancer patients; many of whom receive both myelosuppressive and immunosuppressive drugs. Hence, it is only commonsensical that pretreated patients develop more infections than untreated cancer patients (74).

Table 1 Microbiology and Patient Morbidity and Mortality According to Klastersky, et. al., 2007, of 2,142 Febrile Neutropenic Cancer Patients			
	Total	Complications	Mortality
Bacteraemia	n=499 (23%)*		
Single Gram-negative	168 (34%)	38 (23%)	30 (18%)
Single Gram-positive	283 (57%)	57 (20%)	13 (5%)
Polymicrobial	48 (10%)	11 (23%)	6 (13%)
At least one Gram-negative	29	8	5
Only Gram-positive	19	3	1

* Percentage based on total number of febrile neutropenic cancer patients (n=2,142).

Bacteremia is a leading cause of life-threatening infections in cancer patients receiving chemotherapy (75, 76). In a study of over 2000 neutropenic cancer patients, 23% developed bacteremia. Of these, 57% were from Gram-positive organisms, 34% were Gram-negative organisms and 10% were poly-microbial. These infections resulted in mortality rates of 5%, 18% and 13% respectively, as seen in Table 1 (77). Bacteremia causes higher mortality rates and increase healthcare costs in cancer patients by delaying chemotherapeutics and prolonging hospital stays (78, 79).

In order to help prevent bacteremia, physicians develop prophylactic antibiotic regimens to fight infections before they begin. Cancer patients are predictably neutropenic at specific times of

treatment. Many infections can be expected based on the antibiotics administered and the length of neutropenia as seen in Table 2. The probability of infection increases proportionately as neutropenia worsens and prolongs. Initially, prophylactic antibiotics are administered in attempt to protect a neutropenic patient from the patient's own normal flora. The spectrum of activity in a given antibiotic can allow for prediction of the succeeding infection. Currently, fluoroquinolones with antipseudomonal activity are most commonly used, as they are broad spectrum antibiotics and are useful against endogenous flora found on the skin and in the gastrointestinal tract. These are the predominant sources of infection throughout neutropenia.

Several days post antimicrobial therapy, the reduction in Gram-negative organisms and ongoing neutropenia will outfit for pro-Gram-positive selective pressures and allow for bacteremia by endogenous organisms like *Streptococcus viridans*, multiple species of *Staphylococci*, and *Corynebacteria*. These Gram-positive organisms are found in the normal oral flora and on the skin. When neutropenia persists into a second week, infections are primarily yeasts like *Candida*. Antibiotics against both Gram-positive and Gram-negative organisms promote translocation of overgrown yeast species from the GI tract thus increasing the risk for candidemia (80-82).

The third week of neutropenia consists of higher rates of infections originating in the GI tract, skin, respiratory tract, and nosocomial infections. Molds like *Aspergillus* and *Fusarium* inhaled before neutropenia set in can result in infection at this time. Although hospitals cannot combat molds inhaled before hospital admission, air filtrations systems are used to help reduce the risk of acquisition of these infections from hospital environments. In addition to molds, reactivation of viruses like *Herpes simplex*, *Varicella zoster*, *Cytomegalovirus*, and *Adenovirus* and *BK virus* are also common during this phase of neutropenia. 70-80% of patients with seropositive *Herpes*

simplex will develop an infection which may extend into the esophagus leaving the patients vulnerable to other infections through damaged mucosal membranes (62). Furthermore, multi-drug resistant (MDR) Gram-negative organisms colonizing the GI tract are of concern, as they are the only microorganisms left after treatment with multiple broad spectrum antibiotics and they result in serious infections with increased mortality rates. Recently, drug resistant pathogens accounted for 34% of bacteremia in cancer patients (83).

<u>Week of neutropenia</u>	<u>Antibiotic Use</u>	<u>Resulting Infection</u>
Week one	Gram-negative action antimicrobial	Gram-positive bacteria (i.e. <i>Staphylococci</i> , <i>Streptococci</i> , <i>Corynebacteria</i>)
Week two	addition of Gram-positive action antimicrobial	Yeast (i.e. <i>Candida</i>)
Week three	addition of antifungal	Mold (i.e. <i>Aspergillus</i> , <i>Fusarium</i>)

Lastly, poly-microbial infections must be considered in an immune depleted patient population. Unfortunately, a lack in reporting of these infections has kept them under the radar. Recent data shows that up to 15% of bacteremia in neutropenic patients are poly-microbial. Poly-microbial infections are even more frequent in sites of pneumonia, enterocolitis and peri-rectal infections (77, 84, 85).

Bacterial Infections

Due to constant fluctuation regarding the frequency of Gram-positive and Gram-negative infections in cancer patients, it is safe to deduce that infectious disease patterns vary with respect to geographical location and facility (76, 86). However, a few trends have been perceived in the past decade. Upon initial use of cytotoxic chemotherapy, Gram-negative bacteria, presumably endogenously acquired from the GI tract, were the most common infections (73, 87). Subsequently, Gram-positive organisms became responsible for the majority of infections in neutropenic cancer patients due to Gram-negative directed prophylaxis, central venous catheters, and frequent oral mucositis (76, 88). Recently, non-fermenting Gram-negative bacilli have surfaced in cancer patients, including *Acinetobacter* and *Stenotrophomonas*, along with the advancement of MDR organisms (88-93). Finally, the gradual cessation of fluoroquinolone prophylaxis in some institutions in hopes of prevention of further drug resistance may play a role in the current shift towards dominance of Gram-negative infections (86).

Gram-positive Organisms

In neutropenic patients, the most frequently isolated Gram-positive is coagulase negative *Staphylococcus* (CoNS). These organisms are considered less virulent than most and are commonly found in normal flora. The infections caused by CoNS are usually central-line-associated-bloodstream infections (CLABSI). *Staphylococcus epidermidis*, *S. hominis*, and *S. haemolyticus* and *S. lugdunensis* are the most common CoNS isolated from cancer patients (94). CoNS are also associated with infections in Ommaya reservoirs when accessed numerous times (95-97). Excluding *S. lugdunensis*, these infections are ordinarily treated with antimicrobials and

removal of catheter is sometimes warranted (98). *S. lugdunensis* is more virulent than the former and requires treatment similar to *S. aureus* owing to its increased pathogenicity (99).

S. aureus is a much more serious infection. This is the second most common Gram-positive species isolated from neutropenic patients. On average, 25% of the population is colonized with *S. aureus* in the anterior nares (100). Immunosuppressed patients have higher rates of colonization and infections with significantly high morbidity and mortality. Additionally, some cancers, like Hairy Cell Leukemia, prevent proper functioning of the neutrophils against *S. aureus* (101). If the infection is due to CLABSI, antimicrobials are administered and catheter removal is typically required. Furthermore, evaluations for endocarditis and abscesses are essential as *S. aureus* can swiftly wreak havoc on the heart and epidermis (98).

Furthermore, methicillin resistant *S. aureus* (MRSA) make empirical treatment of Staphylococci infections even more challenging. A study in Korea revealed mortality rates of methicillin-susceptible *S. aureus* (28.8%) compared to MRSA (38.1%) mortality rates with a difference of almost 10% (102). MRSA colonization is increasing which in turn results in higher rates of infection by MRSA (100). MRSA infections have been seen in as much as 55% of Staphylococci infections in cancer patients regardless of neutropenia and treatment failure rates of MRSA in cancer patients can be greater than 50% (103, 104). MRSA accounts for almost a quarter of MDR infections in cancer patients (83). This influx of MRSA infections exacerbates the use of vancomycin for treatment which, in turn, leads to vancomycin resistance (104).

Infections with *Enterococci* in cancer patients usually arise after prolonged antimicrobial therapy with broad- spectrum antibiotics. This alteration in the lower GI tract allows for substantial increase of *Enterococci* which can then cause bacteremia by translocation through the GI tract or

ascending urinary tract infections (105). Unfortunately, increased use of vancomycin due to MRSA infections may play a part in the most recent 15-20% of Enterococci presenting resistance to the drug (106). Patients who have received chemotherapy are at a high risk for development of VRE infections (107). Kamboj published a study in 2010 where 247 patients underwent hematopoietic stem cell transplants (HSCT). VRE was the leading cause of bacteremia 30 days post-transplant, accounting for 53.5% of bacteremia infection with a 9% mortality rate (108). Like MRSA, colonization with VRE can increase likelihood of bacteremia post chemotherapy (108-110). There is a strong correlation between VRE carriage and development of VRE infections, specifically infections of bacteremia (110, 111).

Viridans group *Streptococci* (VGS) is another source of bacteremia in chemotherapy patients. The most commonly found VGS species found in these infections are *S. mitis*, *S. sanguis* and *S. salivarius*. Although VGS are normal in oral flora, mucositis of the mouth may allow for transition into the blood and successive endogenously acquired bacteremia. Therefore, patients on chemotherapy known to cause excessive oral mucositis, like cytosine arabinoside, are at an increased risk for VGS infection (112, 113). Fluoroquinolone prophylaxis may also play a part in encouraging the growth of VGS by destruction of competing organisms. VGS bacteremia can cause rapid sepsis, presenting as hypotension and acute respiratory distress syndrome (ARDS) with a 12% mortality rate (113, 114). VGS are also responsible for streptococcal toxic shock syndrome with a mortality rate between 40-50% regardless of antimicrobial therapy (112-114). Furthermore, recent documentation of drug-resistance is being seen in multiple facilities. MD Anderson Cancer Center reports Vancomycin tolerant VGS (115) and Penicillin-resistant VGS as high as 27% among cancer patients (116). Both Sweden and Spain show similar VGS penicillin resistance of 20-30% (117, 118).

Other, less predominant, Gram-positive organisms known to cause infections in cancer patients, specifically neutropenic patients, include *Bacillus spp*, *Corynebacterium spp*, *Micrococcus spp*. These tend to cause CLASBSI infections but more serious infections have also been documented. *Bacillus cereus* has been isolated in cases of fulminant sepsis and has caused massive intracellular hemolysis due to production of multiple hemolysins (119, 120). Furthermore, *Corynebacterium jeikeiun* has also been reported in cases of sepsis in patients with ALL. Regrettably, *C. jeikeiun* tends to be resistant to multiple antibiotics and, similarly to *C. diphtheriae* in healthy people, *C. jeikeiun* can cause skin and pulmonary lesions in neutropenic patients (121, 122).

Listeria monocytogenes may also cause infection during immunosuppression by violation of the central nervous system (CNS). *L. monocytogenes* has a high mortality rate in immunocompromised patients due to invasion in the CNS resulting in meningitis, brain abscesses, and bacteremia (123, 124).

Gram-negative Organisms

The most common Gram-negative pathogens are endogenously acquired through the GI tract. These pathogens include *Pseudomonas aeruginosa*, *Escherchia coli* and *Klebsiella* which cause between 45-60% of Gram-negative infections (77, 88, 118, 125). Each of these organisms may cause sepsis by endotoxin release in the bloodstream resulting in hypotension, renal failure, and shock. Due to their severity, Gram-negative organisms are usually associated with high morbidity and mortality. Furthermore, the use of antimicrobial prophylaxis in neutropenic patients in order to prevent these infections has resulted in the emergence of significant resistance to quinolones and aminoglycosides among *E. coli* and other Gram-negative species

(126, 127). Carbapenems and fluoroquinolones have been identified as risk factors for drug resistance when used over prolonged periods of time (128).

Despite prophylactic treatment for Gram-negative organisms, *Pseudomonas aeruginosa* is the most repeatedly isolated non-fermenting Gram-negative organism due to its remarkable ability to evade and persist in the GI tract, translocate into the blood, and development of antibiotic resistance by various mechanisms (129-131). Hematological disease is associated with an increased risk for *P. aeruginosa* bacteremia. A recent study of patients with hematological malignancies over a 70 month period, who were prophylactically treated with levofloxacin when neutropenia was expected for greater than 7 days, confirmed 441 bloodstream infections of which, 15% were identified as *P. aeruginosa* and 57.3% were Gram-negative. Additionally, one third of the *P. aeruginosa* were MDR. The thirty day mortality rates for all bloodstream infections, *P. aeruginosa*, and MDR-*P.aeruginosa* were 11.3%, 27.3% and 36.4% respectively (132). In the past decade an increase in fluoroquinolone-resistance has been seen in *P.aeruginosa*. Pakistan reports an increases from 13.3%in 2000 to 29.4% in 2006.

Furthermore, *P.aeruginosa* is the most frequently isolated Gram-negative organism isolated from poly-microbial infections (84). Poly-microbial infections that include *P.aeruginosa* have mortality rates greater than 50% (133). Poly-microbial infections are less responsive to antimicrobial treatments due to their complex nature. They tend to comprise more complicated infections like pneumonia, enterocolitis, and tissue necrosis where deep tissues are involved. Recently rates of poly-microbial bacteremia have been documented in 15% cases involving cancer patients(77).

Escherichia coli are the most prevalent Gram-negative pathogen, with an impressive ability to infect multiple mucosal surfaces and more, it is of no surprise that *E. coli* is a constant threat to all patients. This broad range of infections can include, UTIs, pneumonia, colitis, bacteremia, and so on. Lung cancer patients are at risk for pneumonia due to *E.coli*, these infections have been reported as high as 37.5% (134). However, this great degree of variability in *E. coli* can be even more dangerous in neutropenic patients. Oddity infections, like pyomyositis, have been documented in neutropenic cancer patients (135). Furthermore, *E. coli* seems to infect these patients regardless of targeted antibiotic prophylaxis. Occurrences of bacteremia by *E. coli* have been as high as 37% in some patient populations (106).

E. coli is also the frontrunner in fluoroquinolone resistance, which makes this GNB even more dangerous (136). Variations can be observed based on geographical location and facility but resistance continues to escalate. Kjellander et. al, study indicated no change in Gram-positive and Gram-negative bacteremia ratios of 53.1% and 46.9% respectively even though prophylaxis was halted in an attempt to combat the accumulation of multidrug resistant organisms. *E. coli* accounted for 17.8% of these blood isolates. Furthermore, there was an increase in fluoroquinolone resistance, 2% in 2001 and 16% in 2008, despite the absence of fluoroquinolone prophylaxis at this particular facility in Sweden. Additionally, 36% of patients with fluoroquinolone-resistant *E. coli* died within 30 days (118). A study of hematologic malignancies in Italy treated half of its patients with fluoroquinolone prophylaxis and fluoroquinolone-resistant isolates were found in 81% of patients compared to 37% in the non-treated patients (137). The United States, where quinolones are widely used, also shows increases in fluoroquinolone-resistant *E.coli* (127).

On the other hand, striking decreases of fluoroquinolone-resistant *E. coli* were seen in the cessation of quinolone prophylaxis in Spain, 71% in 1996 to 37% in 2010(117).

Variably, Chong et al, reported no quinolone-resistant *E. coli* during a 2 year time frame of quinolone prophylaxis at a facility in Japan (138). As expected, the percentages of Gram-negative infections at facilities that forgo prophylaxis are increased in comparison to antimicrobial prophylaxis treated patients (92, 138); however, *E. coli* infections specifically do not continually show significant change (117, 138).

Moreover, Extended-spectrum β -lactamase-producing (ESBL) *E. coli* is also an emerging worldwide pathogen and has proven to be particularly unforgiving in cancer patients (139, 140).

In a single center investigation, cancer patients receiving antimicrobials had higher rates of ESBL *E. coli* bacteremia than patients who were not receiving antimicrobials. Furthermore, 12.6% of all *E. coli* bacteremia cases were caused by ESBL *E. coli*. The patients with ESBL *E. coli* also had higher mortality rates than non-ESBL inflicted patients. Analysis of ESBL genes from this study did not support a clonal spread as CTX-M, SHV and TEM were 74%, 19% and 7% predominance respectively (141). A similar study at a single facility in Italy showed higher ESBL *E. coli* prevalence and once again polyclonal distribution was exhibited by TEM, 36%, CTX-M, 33% and SHV, 31% (137). Although some monoclonal nosocomial dispersion has been suggested based on TEM, CTX-M and SHV data, there are other risk factors for acquisition of ESBL *E. coli* bacteremia (138). Previous exposure to fluoroquinolones and carriage of ESBL *E. coli* have been identified as potential risk factors for bacteremia in cancer patients (110).

Klebsiella spp. is consistently the third most prevalent Gram-negative organism isolated from bacteremia in cancer patients and frequently accounts for about 10% of infections in neutropenic

patients (106, 118, 138). Recent attempts to forgo antimicrobial prophylaxis have allowed *Klebsiella spp.*, which colonize the GI tract, to more readily invade and cause infections in cancer patients (138). Unfortunately, *Klebsiella* is also an ESBL-producing bacterium. It appears as though ESBL *Klebsiella* may be acquired in the community as studies show even after a 3-year period of stopped antimicrobial prophylaxis, 14.3% *K. pneumoniae* isolates from infections were ESBL-producers. This is indicative of communal acquisition of isolates which have now colonized the GI tract of patients. These ESBL strains were, like ESBL *E. coli*, predominately CTX-M which seems to be a global commonality (138) .

Although non-fermenting Gram-negative bacilli (NFGNB) are less frequently encountered they are making notable increases in the proportion of Gram-negative infections among cancer patients specifically with regard to *Pseudomonas aeruginosa*, *Acinetobacter spp.*, and *Stenotrophomonas maltophilia* (142). *P. aeruginosa* makes up a large part of the 40% of Gram-negative infections caused by NFGNB. It is the most frequently isolated NFGNB as it accounts for approximately 15-20% of Gram-negative infections in cancer patients. *P. aeruginosa* is not a novel problem and it has been seen in severe infections for decades (132, 143). Conversely, *S. maltophilia*, have not been seen as often in the past and are currently making headway in hematological cancer patients. This NFGNB is frequently multi-drug resistant and is not susceptible to fluoroquinolones that are commonly used in cancer patients making it incredibly dangerous under these circumstances (144, 145).

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CHAPTER TWO:

**MOLECULAR EPIDEMIOLOGY OF *ESCHERICHIA COLI* CAUSING
EXTRAIESTINAL INFECTIONS IN PATIENTS WITH CANCER**

Introduction

Phylogeny of Escherichia coli

The species *Escherichia coli* is a prominent member of the *Enterbacteriaceae* Family. With an assortment of variable strains, *E. coli* plays a major role in both health and infection of the human body. Commensal strains of *E. coli* are best known for their role as major facultative anaerobes of intestinal microflora. They colonize hours after birth and thrive with upwards of 500 other bacteria in the mucosal lining of the colon. Here they persist for the remainder of life and aid in protection, digestion and absorption in the gut (1). Survival in this competitive environment of various organisms and host interactions requires significant ability to adapt. The ability of *E. coli* to acclimate under selective pressures is what has led to the great divide in commensal and pathogenic isolates. *E. coli* uses a multitude of metabolic and regulatory mechanisms to adjust to countless environmental stressors (2-6).

The main pathogenic types of *E. coli* include intestinal pathogenic *E. coli* (IPEC) and extraintestinal pathogenic *E. coli* (ExPEC). These can be further delineated based on pathogenic traits as enteroaggregative *E. coli* (EAEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic

E. coli (ETEC), enteroinvasive *E. coli* (EIEC), enteropathogenic *E. coli* (EPEC), diffusely adherent *E. coli* (DHEC) and adherent-invasive *E. coli* (AIEC), which are generally categorized under IPEC. While ExPEC have further defined pathotypes of meningitis-associated *E. coli* (MNEC), septicemia-associated *E. coli* (SEPEC), urinary tract infection or uropathogenic strains (UPEC) and avian pathogenic *E. coli* (APEC)(2, 3, 7).

Deletion and acquisition of DNA is a critical part of prokaryotic evolution and the plasticity with which *E. coli* is able to accomplish this is largely what makes it such a successful microorganism. Thus, the various pathogenic strains of *E. coli* do not have a single evolutionary origin within *E. coli* but have risen numerous times. Strains can acquire the appropriate virulence factors to give rise to a pathogenic form (8). *E. coli* was one of the first organisms used to display the importance of these exchanges as it maintains the ability to laterally acquire and transfer genes in order to promote survival in the host. Multi-locus enzyme electrophoresis (MLEE) work by Selander and Whittam demonstrates that although the single locus-diversity estimates suggest neutral gene theory, combinations of alleles into electrophoretic types indicate genetic differentiation into distinct groupings by particular genetic combinations via selective differences (9). Furthermore, acquired virulence factors, which allow the cell to adhere to tissue surfaces, evade the immune system and disseminate through the body are also associated with pathogenic *E. coli* (10-13). Therefore, each of the classifications of *E. coli* pathotypes is based on phylogenetic background, virulence factors and the obvious clinical manifestation of disease.

An *E. coli* (ECOR) reference collection established by Whittam, Ochman and Selander was originally used to phylogroup by MLEE (9). This classification is largely true today and is currently used as a reference tool to designate isolates into one of the four major lineages, A, B1, B2, D. Commensal strains belong mainly to phylogenetic groups A and B1, are devoid of most

virulence factors, they are sister taxa. Some strains of A, B1 but more so D, are pathogenic and often maintain an increased number of virulence factors. However, commensal strains can be isolated from pathogenic conditions suggestive of host-dependent factors like immunosuppression from underlying disease. Pathogenic *E. coli* found in infections of healthy individuals is most frequently associated with phylogenetic groups B2 and D. B2 is a divergent lineage associated with increased virulence and high mortality upon in vivo challenge (14).

Furthermore, increases in MLST data sets and further characterization of a greater number of strains have evolved to document ancillary phylogroups E,C, and F. Phylogroup E, which was the first of these recognized accessory groups, is now well characterized and contains the famous O157:H7 pathogenic EHEC strain. Phylogroups C and F are closely related to B2 and B1 phylogroups respectively (1). There is an apparent interdependency of pathogenicity and metabolic activities displaying by correlation of phylogeny and pathogenicity (15).

The Problem of ExPEC

E. coli has the ability to infect and disseminate through every mucosal surface in the human body (16). Due to a unique capability to adapt to a multitude of environments, ExPEC is the single most prevalent pathogen for all urinary tract infection syndromes, resulting in upward of 1.5 billion dollars in healthcare costs a year. Each year *E. coli* is responsible for over one hundred thousand cases of sepsis, approximately forty-thousand sepsis-related deaths and tens of thousands of cases of pneumonia (17). Furthermore, treatment of *E. coli* infections with standard antibiotics is increasingly ineffective due to drug resistance of the adaptive organism. This drug resistance in *E. coli* is associated with higher mortality rates in patients (10).

Recent reports of clinical ExPEC investigations demonstrate variability of *E. coli* to be extraintestinally infective even though the phylogroup it belongs to may not characteristically be of a pathogenic origin (18). Extraintestinal *E. coli* strains frequently originate from the intestines and are physiologically fit for invasion outside of the gut. This invasion results in endogenously acquired bacteremia. The variability of these phylogenetic relationships and virulence factors throughout ExPEC strains can allow for novel sequence types and dissimilar virulence patterns resulting from selective pressures in the environment.

ExPEC in Cancer Patients

Depleted immunity and extended hospital visits make cancer patients inordinate hosts for the acquisition of novel pathogenic infections. Chemotherapy, solid tumor cancers and prior antibiotic usage have been identified as risk factors for extraintestinal *E. coli* infections (19-22). The alterations of environment in cancer patients, such as chemotherapy treatments, radiation, drugs and prophylactic antibiotic treatment, could very likely allude to a rapid evolutionary pattern in *E. coli* genotypes, resistance patterns, and virulence factors in ExPEC infections.

Ironically, the same interventions used to treat cancer are also variables which increase risks of bacterial infections. These infections interfere with chemotherapy treatment in cancer patients and are greatly associated with increases in morbidity and mortality. Although some risk factors are known, there is a critical lack of knowledge in the types of *E. coli* infecting cancer patients. For example, in 2010 M.D. Anderson Cancer Center linked *E. coli* with deadly pyomyositis infections. In a normal patient population, ninety percent of pyomyositis infections are caused by Gram positive organisms. Furthermore, the oddity Gram negative pyomyositis infections in this population are largely harmless. Conversely, in cancer patients with hematologic

malignancies, a 33% mortality rate was documented and molecular analysis of the 6 available isolates proved 5 to be from the same lineage, ST131 (11). This lethal sequence type is implicated in extended-spectrum beta-lactamase production which results in increased antibiotic resistance. As this type of infection is uncommon in a healthy patient population and empirical treatment is the most influential variable in mortality rates of cancer patients, characterization of extraintestinal *E. coli* isolated from cancer patients by genotypes is critical to understanding the ExPEC infections debilitating these patients (22).

More recently, a significant shift from Gram positive to Gram negative bacterial infections has been documented in patients with cancer. In 2008 a large-scale association between cancer patients and multi-drug resistant *E. coli* was reported during a 16 month screen. 823 patients from a cancer center in Italy demonstrated a significant change in the types of bacteria infecting cancer patients with hematological malignancies. The majority of isolates were Gram negative and a quarter of all specimens were *E. coli*. Hospital protocol prophylaxis employed fluoroquinolone antibiotics if neutropenia was present for greater than seven days. 87% of the ExPEC from these patients was found to be resistant to fluoroquinolone and 97% of patients on prophylaxis presented with fluoroquinolone resistant ExPEC. Moreover, of patients who did not receive prophylaxis, only 44% were infected with fluoroquinolone resistant extraintestinal *E. coli*.

In 2009, another correlation was found between ExPEC infections and patients with cancer at MD Anderson Cancer Center. Cancer patients were inundated with multi-drug resistant *E. coli* in cases of bacteremia. This resistance was thought to be selected for by prophylactic treatment with fluoroquinolones which then allowed for explicit fluoroquinolone resistance types of *E. coli* to persist in these patients. A similar consequence of bacterial infections in allogeneic

hematopoietic stem cell transplant recipients at Mayo Clinic was documented from 2003-2008 when prophylaxis entailed levofloxacin and either penicillin or doxycycline. While Gram negative organisms did not account for a single infection in 2003, Gram-negatives progressively escalated to 46% in 2008. Of those, multidrug resistance was seen strictly in the classes of drug for which patients were prophylactically treated (19).

Insufficient treatment of Gram negative infections has resulted in a change in the epidemiology and antibiotic resistance of previously easily treated infections (21). In patients with cancer, initial accurate treatment is crucial to the outcome of infection. A single study reported a mortality risk ratio from multi-drug resistant *E. coli* greater than 6 times that of non-multi-drug resistant isolates from the same facility. Increases in drug resistance play a significant role in the inability to cure infections, in turn, increasing morbidity and mortality rates in cancer patients (22).

Unfortunately, we have yet to expose all of the risk factors of cancer and cancer therapy due to a lack of comprehensive knowledge regarding the ExPEC infections and how they correlate to cancer and cancer treatment. Steps must be taken to identify high risk cancer patients and discernible flags from these types of *E. coli* infections must be sought out for improved prevention and management of ExPEC infections in cancer patients. Evidence shows that adequate empirical antibiotic treatment plays a critical role in the outcome of infection. A study to assess antibiotic resistance, adequacy of initial antibiotic therapy and patient mortality of specimens from 1997-2005 at Sierrallana Hospital, in Spain inferred multidrug resistance played a significant role in incorrect empirical antibiotic treatment and patients with MDR *E. coli* had significantly higher mortality than non-MDR *E. coli* hosts (22).

A retrospective, double matched, case-control study in 2008 resolved that statistically significant risk factors for bacteremia with multi-drug resistant *E. coli* in cancer patients are chemotherapy, surgery and radiation within 30 days prior to infection. Cancer patients undergoing these procedures commonly receive prophylactic antibiotic treatment. The study also suggests that these two factors may result in multi-drug resistant *E. coli* in the bloodstream because of mucosal upset of native microflora (19). A sixteen year study at Rabin Medical Center in Israel proved that resistance of the Gram-negative bacteria to broad-spectrum beta-lactams used to treat febrile neutropenia, a common ailment in cancer patients, increased with length of hospital stay prior to onset of bacteremia increasing from 8% acquired before hospitalization to 48% when acquired 14 days after admission. Similar resistance trends were seen in the children's hospital with length of hospital stay. More individualized approaches are necessary based on antibiotic exposure and other risk factors like length of hospital stay (20).

In cancer patients, the problem of ExPEC is exaggerated. *E. coli* infections interfere with chemotherapy treatment, increase patient mortality rates and healthcare costs (23). The circumstances surrounding these patients make them inordinate hosts for development and transmission of resistant organisms, yet there is a gap in knowledge about the specific types of *E. coli* affecting patients with cancer.

Aims

Aim 1: Define the genotypes and clonal groups attributable to ExPEC infections in patients with cancer from H. Lee Moffitt Cancer Center (MCC).

Multilocus sequence typing (MLST) will be used to assign genotypes to clinical and control isolates of *E. coli*. The genetic relatedness of MCC and to reference control

collections will be determined using molecular phylogenetic analyses. MLST will be used to model the evolutionary history, population structure and patterns of dissemination of *E. coli* causing extraintestinal disease at MCC.

Aim 2: Determine the antibiotic resistance and virulence attributes of ExPEC infections in cancer patients.

Antibiotic susceptibility testing will be performed using disk diffusion. Multiplex PCR will be used to define the profile of virulence factors associated with extraintestinal disease in MCC *E. coli* isolates compared to control strains.

Results

Patient Population

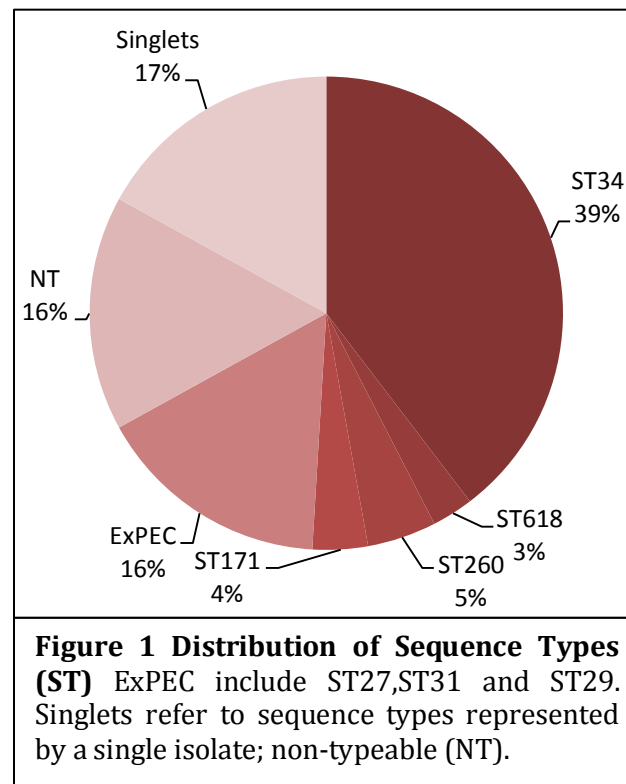
Site of Infection	Female	Male	Unknown*	Total Infections	% Total
Wound	6	8	1	15	13%
Blood	4	8	3	15	13%
Urine	44	22		66	58%
Abdominal Fluid	5	6		11	10%
Other	3	1		4	4%
Sputum	0	3		3	3%
Total	62	48	4	114	100%

*Unknown gender information resulted from damage to isolate label.

114 cases of ExPEC infections were collected from MCC between 2009 and 2013. Most patients were female (56% of cases). The average patient age was 61 with the youngest patient being 22 and the oldest 90. There was no significant difference in average age of infection between genders. Table 1 shows population gender and sites of infection. The most common site of infection was the urinary tract followed by the blood and wounds.

MLST Analysis

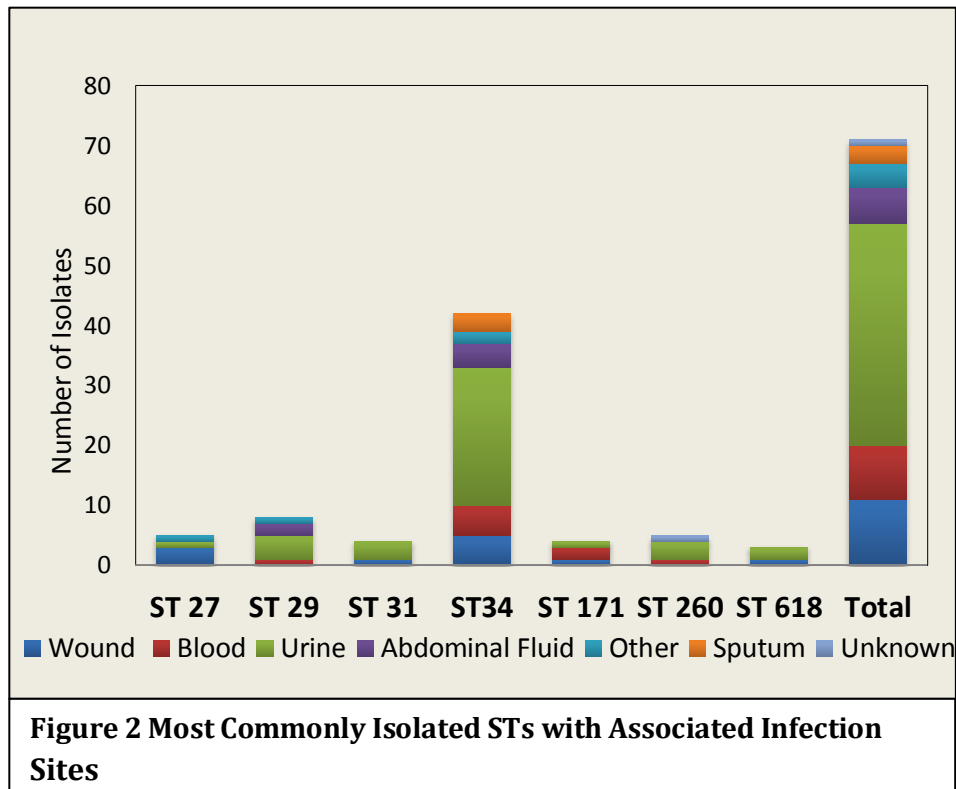
DNA sequencing via MLST allowed for direct assignment of multiple housekeeping alleles. The 5' ends of seven housekeeping genes (*aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh* and *uidA*) were sequenced and consensus sequences were assembled for genes in each of the MCC isolates. Sequence comparisons among 105 isolates revealed 24 distinct combinations, not including 17 non-typeable isolates. Non-typeable isolates contain MLST profiles not found in the EcMLST database.



Among the 24 distinct sequence types (ST) there were 17 singletons and 7 STs isolated more than once. Distribution of common STs is seen in Figure 1. Classic extraintestinal *E. coli* (ExPEC) (ST27,ST31 and ST29) make up only 16% of total isolates. ST27 and ST31 are uropathogenic genotypes, while ST29 is a neonatal meningitis genotype. ST171 is characteristically a Shiga-toxin producing (STEC) genotype, whereas ST34, ST260 and ST618

are genotypes associated with enteroaggregative *E. coli* (EAEC).

The most commonly isolated STs (three or more times) are seen in Figure 2. These isolates include ST27, ST29, ST31, ST34, ST171, ST260 and ST618. They make up 67.6% of total isolates and are found in each of the sites of infection. ST34, was the overwhelmingly the most frequently isolated ST and occurred in every site of infection. Half of ST171 and related NT isolates were found in the blood. The urinary tract was the site of infection for more than half (58%) of the most common STs seen here and a quarter of the remaining common STs are from wound (14%) and blood (10%) infections.



Phylogenetic Analysis

Commensal and pathogenic *E. coli* strains can be divided into four major phylogroups referred to as the ECOR A, B1, B2 and D groups (24). Typically commensal strains belong to groups A and B1, whereas *E. coli* associated with ExPEC are found in B2 and to a lesser extent D. To

determine the distribution of ECOR phylogroups among ExPEC infecting cancer patients we compared strains with known ECOR reference strains as seen in Figure 3. A MUSCLE alignment was used to construct a Neighbor-joining tree through MEGA5.05. MCC isolates cluster into six clonal clusters (Clonal Clusters I-VI). Bootstrap values, indicated at nodes, were used to define clonal clusters when values were greater than 70%. Moffitt isolates are indicated by MCC numbers, clinical reference strains are denoted by TW numbers, and phylogroup reference strains are indicated by ECOR numbers. These clusters show a lack of classic extraintestinal pathogenic *E. coli*. Only 22% of these isolates cluster with the phylogroup B2, seen in Clonal Clusters II-IV, strains typically known to cause extraintestinal infections. Instead, the majority cluster north of B2 isolates. This outlier of B2 phylogroup contains ST34 representing 39% (42 isolates) of the total isolates from Moffitt patients. This particular sequence type does not cluster with any of the recognized A, B1, B2, or D phylogroups.

SNP ST131 PCR

Based on the location of ST131 control strains in the cluster analyses, PCR was used to determine if ST34 by EcMLST MLST method is ST131 by Achtman MLST method as conducted in “ST131 *E. coli* SNPs PCR Protocol: Johnson Lab protocol/Brian Johnston, 2008” and optimized for Riordan Lab. SNP specific PCR products in *mdh* and *gyr* genes are associated exclusively with ST131 strains as seen on the Achtman MLST website: <http://mlst.ucc.ie/mlst/>. Of the ST34s analyzed, as seen in Figure 4, 90% were positive for SNP specific PCR products in *mdh* and *gyr* as well as the *uidA* control.

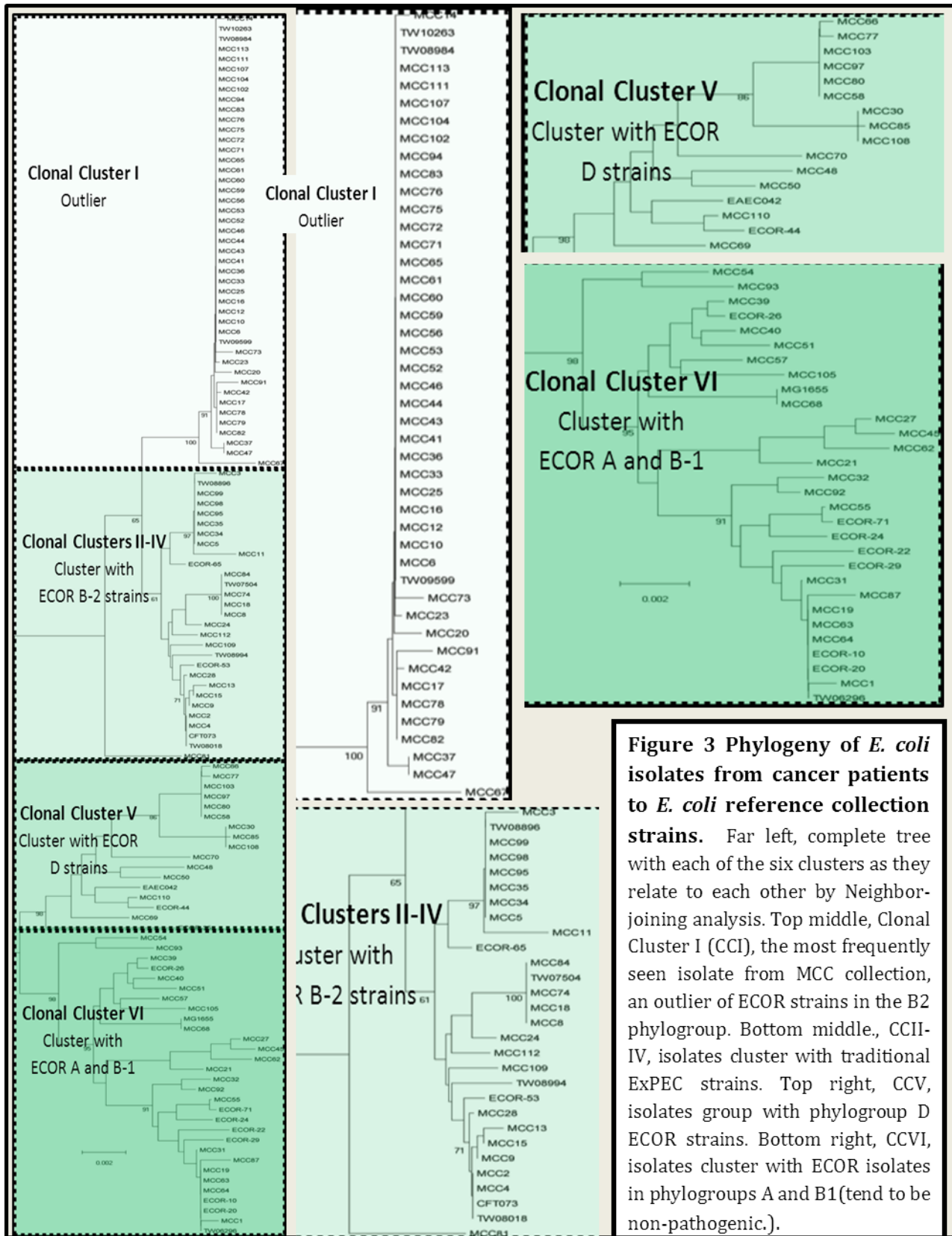
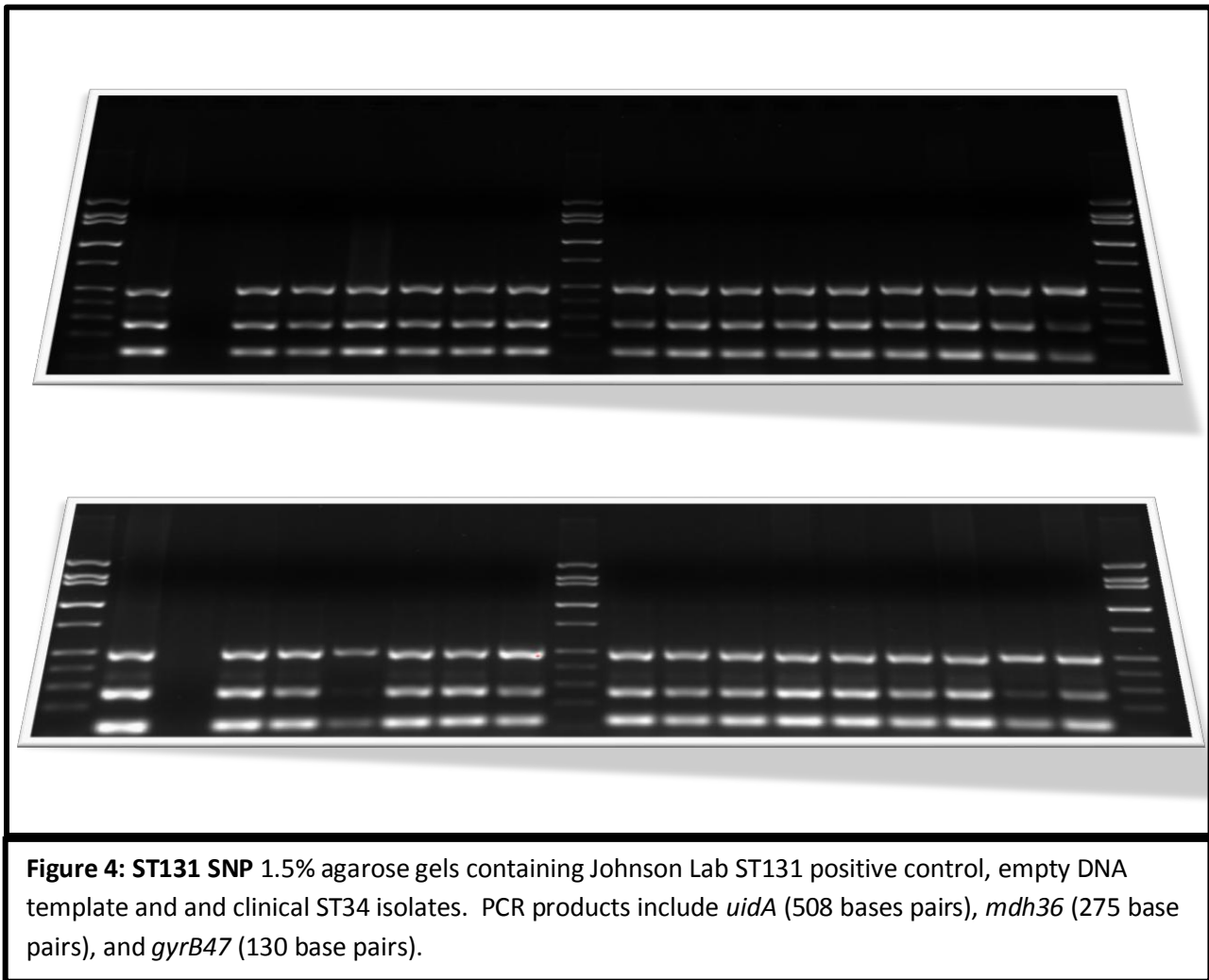


Figure 3 Phylogeny of *E. coli* isolates from cancer patients to *E. coli* reference collection strains. Far left, complete tree with each of the six clusters as they relate to each other by Neighbor-joining analysis. Top middle, Clonal Cluster I (CCI), the most frequently seen isolate from MCC collection, an outlier of ECOR strains in the B2 phylogroup. Bottom middle., CCII-IV, isolates cluster with traditional ExPEC strains. Top right, CCV, isolates group with phylogroup D ECOR strains. Bottom right, CCVI, isolates cluster with ECOR isolates in phylogroups A and B1 (tend to be non-pathogenic.).

RAPD Analysis

RAPD-PCR was employed to assess the clonality of the most common ST, ST34. Visual comparisons of randomly amplified banding, seen in Figure 5, does not suggest an exact clonal match but instead appears to be multiple fingerprints suggestive of variable strains of ST34. Additionally, due to the similarity of determinative SNPs with ST131, ST131 control strains,



were used and again suggest possible disparities in ST by various MLST methods as they also cluster closely with ST34 in RAPD analysis.

Antimicrobial Susceptibility

As seen in Figure 6, Ciprofloxacin, a commonly used fluoroquinolone, proved to be the least successful antimicrobial tested, as 53% of Moffitt isolates are not treatable with the drug. Ciprofloxacin resistance is frequently seen in the healthcare setting (25). Additionally, trimethoprim-sulfamethoxazole was unsuccessful in 39% of ExPEC isolates at Moffitt. Imipenem and cefipime (both cephalosporins) showed the greatest counteractive response against MCC isolates. Of 113 specimens tested for drug resistance, 23% display potential for multidrug resistance, which is defined by resistance to three or more drugs each with a dissimilar mechanism of action. Furthermore, 5% of the isolates show potential for Extended-spectrum beta-lactamase production via resistance to extended-spectrum cephalosporins, ceftazidime and ceftriaxone. One isolate was rejected for antibiotic profiling due to inability to confirm patient data attached to it.

Virulence Profiling

The distribution of virulence factors was evaluated using multiplex PCR. Virulence factors assessed included adhesins, specifically, P fimbriae (*papA*), S and F1C fimbriae(*sfa*, *focG*), and type 1 fimbriae (*fimH*), additionally, capsule synthesis (*kpsMT*), iron scavenging systems (*iutA*), toxins to include hemolysin and shigatoxin (*hlyA*, *stx2A*), and serum survival factor (*traT*). Previous work shows specialization of these virulence factors implicated in ExPEC *E.coli* and lacking in commensal *E.coli* isolates (14).

Well-characterized *E. coli* strains were used for both positive and negative controls. The first 8 of these are commonly seen in extraintestinal *E. coli* while the last two factors are more frequently expressed in enterotoxigenic *E. coli* and other enteric *E. coli* strains. The uncommon

B2 isolates in this patient population had an average of 3 VFs per profile and the non-B2, ST34s, also had an average of 3 virulence factors per profile. More specifically, the ST34 profiles consisted of 97.5% *fimH*, 85.4% *iutA*, and 90.2% *traT*.

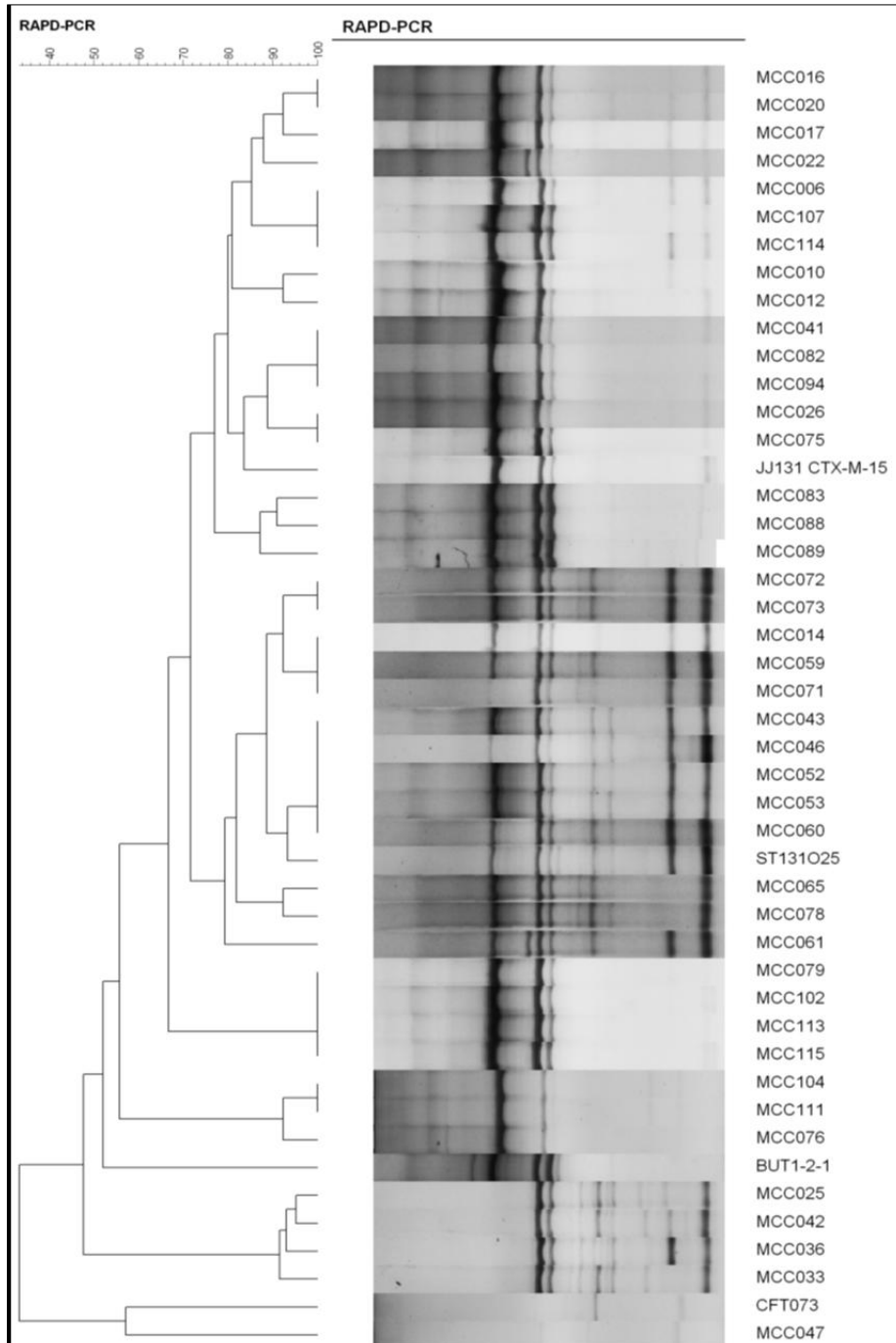


Figure 5 Bionumerics RAPD analysis of clinical ST 34 strains using DICE Similarity Coefficient with UPGMA Clustering. ST131 control strains BUT-1-2-1, ST131O25, JJ131CTM-M-15; as well as the CFT073 UPEC.

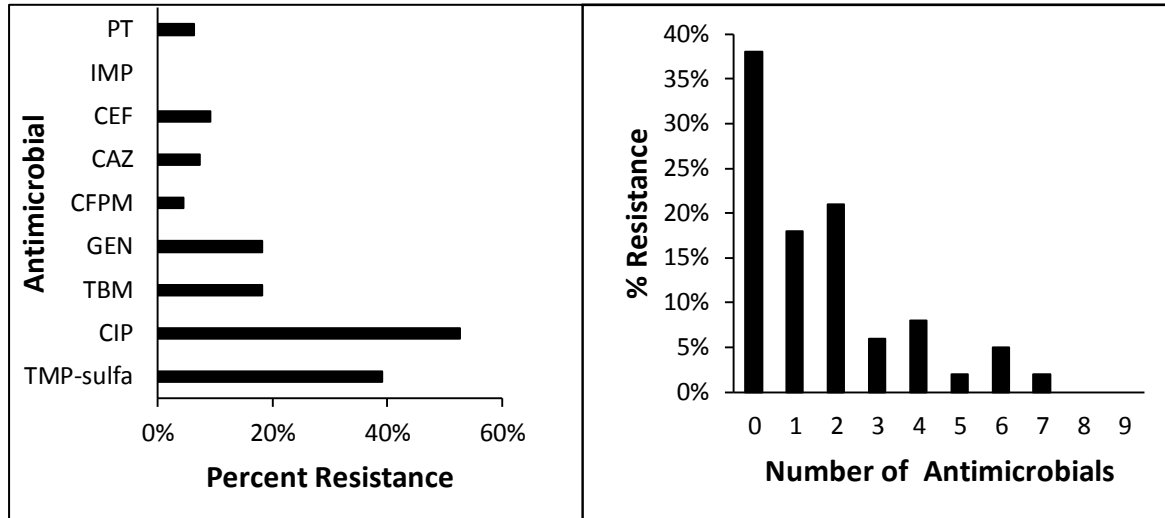


Figure 6 Antimicrobial Resistance of MCC Isolates Piperacillin-tazobactam(PT); Imipenem(IMP); Ceftiaxone(CEF); Ceftazidime(CAZ); Cefepime(CFPM); Gentamycin(GEN); Tobramycin(TBM); Ciprofloxacin(CIP); Trimethoprim-sulfamethoxazole(TMP-sulfa)

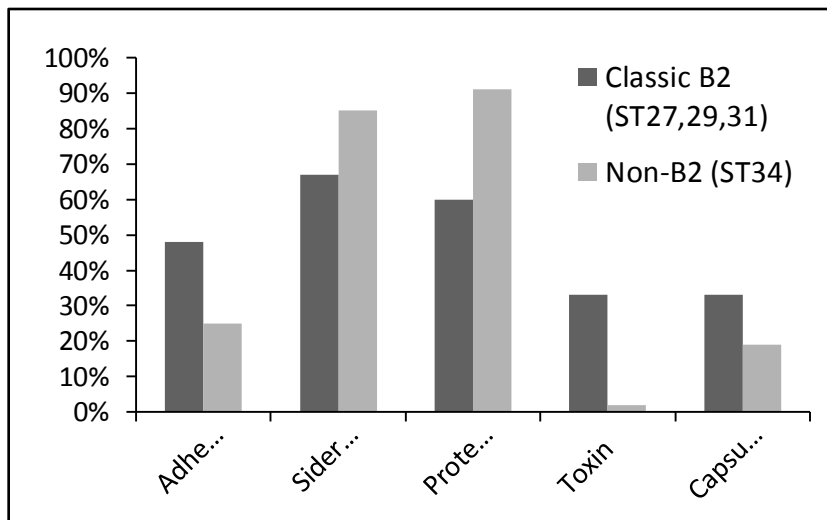


Figure 7 Comparison of MCC B2 and non-B2 Virulence
 Adhesions :fimH, focG, papA, sfa; Siderophore:iutA; Protectin: traT; Toxin: hlyA; capsule synthesis: kpsMT

Although the B2 phylogroup had increased adhesions, toxins and capsule synthesis, the pathogenic ST34 group had greater siderophores and protectins, see Figure 7. Furthermore, in comparison, the virulence potential for clonal cluster 1, containing the ST34, was roughly three. Classic pathogenic B2-clusters exhibit about the same virulence potential as these un-common

outliers of B2, as seen in Table 2.

EAEC-PCR

Type-specific PCR targeting the pAA plasmid was performed on all ST34 isolates using pCVD432 primers with EAEC042 and JJ055 controls. No amplification resulted for any of the ST34 strains from the study.

Table 4: Clonal Cluster Characteristics							
Cluster	Phylogroup	Infection site:					VF Index^a
		Blood (%)	UTI (%)	Wound (%)	Sputum (%)	Other(%)	
I	UG ^b	14	57	18	5	6	2.82
II	B2	4.5	50	36	0	9.5	3.14
III	D	7	62	31	0	0	2.46
IV	A	6	67	22	0	5	1.39

^aVirulence factor index (VF index) is the average number of VFs per isolate.

^bUG (ungrouped) cluster I isolates did not group with any ECOR strains in preliminary analysis.

Discussion

Previous studies by Selander and Whittam suggest that differentiation in electrophoretic types are associated with genetic differences into distinct groups, of which, some show a propensity to cause disease in the gastrointestinal tract due to specific virulence factors and the ability to create a definite fitness niche. This determination permitted the site specific naming of many pathotypes, like IPEC and ExPEC. However, our investigation of sequence types associated with ExPEC infections in cancer patients has brought to light some possible variation in roles of previously characterized “enteric pathogenic *E. coli*”.

Genotypic analysis identified a novel predominating sequence type unassociated with conventional ExPEC infections, ST34. ST34 is an infrequently found ST related to enteroaggregative *E. coli* (EAEC). EAEC is most commonly associated with diarrheagenic

disease of acute and chronic nature and is independent of the level of economic development (26, 27). The EAEC pathotype is heterogenic in nature and is defined by a “stacked brick” adherence pattern to epithelial cells (28). Currently, the most accepted method for EAEC detection is a HEp-2 adherence assay. Probing for genetic markers containing virulence genes like the *pAA* plasmid is also used to identify EAEC. Unfortunately, not all diarrheagenic strains of *E. coli* contain the *pAA* plasmid therefore EAEC is not exclusive to this mobile element (29, 30).

Based on the pCVD432 probe for the *pAA* plasmid, our ST34 isolates do not catalog with *pAA* positive EAEC isolates (data not shown). However, their phylogenetic grouping suggests relatedness to clinically described EAEC diarrheagenic isolates from the Whittam Lab. HEp-2 adherence assays are necessary to further characterize this ST34 group of clinical isolates.

However, based on RAPD-PCR fingerprinting and SNP-ST131 PCR, ST34 isolates are strikingly similar and may be equivalent to ST131 by other MLST methods. This is a forthcoming ExPEC clone described to possess both high virulence potential and antibiotic resistance in many countries (31). ST131s most frequently belong to phylogroup B2 while our ST34s cluster appear to fall outside of the B2 phylogroup and is associated with a clinical enteroaggregative *E. coli* opposed to ExPEC (32). Yet, EAEC strains most commonly cluster in phylogroup D, as did our EAEC042 control strain (33).

ST34 isolates seen in MCC patients clearly have the ability to cause infection outside of the gastrointestinal tract, especially in immunocompromised hosts. Based on our panel of virulence factors specific to ExPEC *E. coli*, ST34 isolates have the virulence potential just as significant as the pathogenic B2 isolates. Furthermore, the prevalence of *iutA* and *traT* genes is blatantly higher in the ST34 cluster when compared to the B2 phylogroup from this study. Previous

studies have revealed that these two virulence factors are higher in ESBL-producing *E. coli* isolates (34). Although, ESBL production was suspect in many of our isolates, PCR confirmation is still essential for future studies.

The characterization of the antibiotic profiles may lead to enhanced primary treatment of extraintestinal *E. coli* infections in cancer patients. The excessive presence of ciprofloxacin resistance (53%) may warrant consideration of various other prophylaxis measures in cancer facilities. Based on the established trends, these variables which allow for pathogenicity of the organism, may be used as predictive markers for infections and allow for a more efficient manner of treating patients at Moffitt Cancer Center. Should these types of *E. coli* exist unilaterally in cancer patients at multiple facilities, the study of these correlations to cancer therapy has the potential to impact the entire medical community by altering prevention and treatment protocol for patients suffering from both cancer and extraintestinal *E. coli* infections.

Furthermore, clustering indicates the ST171 and closely related non-typeable (NT) isolates, predominately isolated from blood, are closely related to the clinical STEC strain, TW06296. This STEC strain is typed as a ST171 and possesses the Shiga toxin gene *stx2AB*. We have found no documentation of dissemination into the blood from this STEC. However, MCC ST171 and closely related NTs may show proclivity to disseminate into the blood via an unknown fitness niche in cancer patients, as five of the seven were recovered from this source. These isolates showed little similarity in antibiotic resistance and virulence factor profiles. The clustering of ST171s with clinical STEC warrants further investigation into the interworking of this sequence type.

Characterization of these isolates' virulence factors may also bring forth patterns of commonly

perceived virulence genes providing a target for rapid PCR detection in order to direct initial antimicrobial treatment. For example, ST34 have a high rate of ciprofloxacin resistance and 90% of isolates contain the virulence factor *traT*. Significant associations have also been made between specific virulence factors and weakened immunity which further commands the need for investigation into the parallels of virulence factors and cancer therapy, as this was not considered previously (14).

Experimental Methods

Clinical Isolates and Cultures

ExPEC isolates were clinically confirmed by Moffitt Cancer Center laboratory personnel. Isolates were streaked to Tryptic Soy Agar slants and transported to the Riordan Lab on ice and labeled sequentially in order of collection. Slants were plated to Luria Broth (LB) Agar (1.5%) and MacConkey Agar plates respectively; then incubated at 37°C for 18 hours. Upon confirmation via phenotypic single organism morphology, a negative Gram stain result and positive lactose fermentation on MacConkey Agar, single colonies were taken from corresponding LB Agar plates and overnight cultures were made using a 1:10 ratio of media-to-flask volume and incubated at 37°C on a rotary shaker (200RPM). Aliquots were prepared with LB containing %15(v/v final) glycerol and stored at -70°C.

DNA Extraction

DNA extractions were taken from single colony isolates that had been inoculated into 10ml Luria Broth and incubated, while rocking, at 37°C until exponential growth phase. As advised by Genra Puregene DNA Extraction Protocol, 1ml of culture was centrifuged at 15000rcf for 5 seconds and supernatant was discarded. This was repeated for a second ml of culture. 600µl of

Cell Lysis Solution was added to the bacterial pellet and mixed by pipetting. This tube was then incubated at 80°C for 8 minutes to lyse the cells. Upon visual clearing of the mixture, 3µl of RNaseA solution was added to the tube, inverted 25 times, and incubated at 37°C for 45 minutes. The sample was then immediately transferred to ice for 2 minutes. 200µl of Protein Precipitation Solution was added to the solution and vortex for 20 seconds. Finally the samples were centrifuged for 3 minutes at 15000 rcf to produce a protein pellet at the extremity of the tube. Supernatant was removed and DNA was precipitated using 600µl 100% isopropanol and inverted 50 times. Globular DNA was visualized and tube spun 15000rcf for 1 min. 600µl 70% Ethyl alcohol was added to the pellet and inverted 50 times, then centrifuged 15000rcf for 1 min. Supernatant was removed by pipetting and tube was inverted to allow drying of ethyl alcohol. Finally, 50µl of autoclaved distilled deionized water was pipetted into the tube in order to lift pellet into solution and 2 hours incubation at 65°C successfully triggered DNA into solution.

Multilocus Sequence Typing (MLST) and Phylogenetic Analysis

Sequencing methodology used for MLST was carried out as part of a system described in detail at <http://www.shigatox.net/new/tools/ecmlst.html>. In total, 7 housekeeping genes (*aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh*, and *uidA*.) were analyzed at various positions around the *E. coli* chromosome (35). Primers used to amplify these loci were obtained from a publicly available database at Michigan State University (MSU; www.shigatox.net) and optimized for the Riordan Lab. Upon electrophoretic confirmation, remaining PCR product was purified using QIAquick PCR Purification Protocol and sent to MWG Operon.

Once sequenced through MWG Operon by dideoxy sequencing, a pairwise analysis was constructed of raw data with MEGA5.05 software. Each trimmed sequence was aligned with K12 reference strain alleles and then compared to sequences in the EcMLST database. Alleles

are assigned based on DNA sequences. Genotypes are assigned based on allele assignment. This data was subsequently used to determine the genetic relatedness of Moffitt isolates to one another by phylogenetic inference and then compared to well-characterized strains. If strains were absent from the database they were considered new sequence types and added to the database. A Neighbor Joining algorithm available through MEGA5.05 was performed on concatenated MLST DNA sequence data with bootstrap confidence values (1000 replications). STs which shared >70% bootstrap support were defined as discrete ST clonal complexes.

RAPD PCR

Random amplified polymorphic DNA (RAPD) fingerprints were generated for ST34 isolates, in duplicate, using the arbitrary primer 1283 to assess the clonality of the ST (36). Reactions were performed in 25 µl volumes containing 25ng of *E. coli* genomic DNA and 25pmol of primer 1283. Amplification in a thermal cycler included an initial denaturation of 5 minutes at 95°C followed by 45 cycles at 95°C, 36°C, and 72°C for 1 minute, 1 minute and 2 minutes respectively. Amplified products were separated by electrophoresis on a 1.5% agarose gel. These patterns were visually compared and transferred into BioNumerics® for analysis. Similarity in banding was compared using the Dice Similarity Coefficient with 1.25% optimization and 1.25% tolerance. Dendrograms were then constructed according to UPGMA analysis.

ST131 E.coli SNPs PCR

PCR was used to determine if ST34 *E.coli* by EcMLST method sequence types as a ST131 by Achtman MLST. Concentrations and thermocycler settings were optimized for the Riordan Lab using a Johnson Lab protocol outline and primers in Table 3. ST131 O25B:H4 was use as a

positive control.

Primer	Sequence	Product Size (bp)	Lab
<i>uidA F</i>	GCGTCTGTTGACTGGCAGGTGGTGG	508	Johnson Lab
<i>uidA R</i>	GTTGCCCGCTTCGAAACCAATGCCT		
<i>mdh36 F</i>	TAACGTTAACGCCGGT	275	Johnson Lab
<i>mdh36 R</i>	GGTAACACCAGAGTGACCA		
<i>gyrB47F</i>	CGCGATAAGCGCGAC	130	Johnson Lab
<i>gyrB47R</i>	ACCGTCTTTTTCGGTGGAA		

Antibiotic Resistance

Antibiotic resistance profiles were evaluated by standardized disk diffusion according to Clinical Laboratory Standards Institute. Nine clinically relevant antibiotics were used to assess susceptibility/resistance of each isolate. Extended-spectrum Beta-lactamase production was confirmed by correlation with patient medical chart data for clinical microbiology laboratory for evidence of resistance to first and second generation cephalosporin in disk diffusion.

Virulence Profiling

Multiplex PCR

Multiplex PCR evaluated the distribution of virulence factors. Virulence factors assessed included adhesion factors (*fimH*, *focG*, *papA*, *sfa*), capsule synthesis (*kpsMT*), iron scavenging systems (*iutA*), toxins (*hlyA*, *stx2A*), and serum survival factor (*traT*) as described by Johnson et. al. (11-14). Primers were validated individually using template DNA from well-characterized

E. coli strains for positive and negative controls. *hlyA* was assessed in a single-plex reaction for undergraduate training. Upon confirmation, pooling of primer optimization was assessed by primer compatibility and product size with relevant DNA template on electrophoretic gel. Final two pools can be seen in Table 6. Gel electrophoresis was used to confirm the quality of each multiplex reaction (Figure 8).

Table 6: Multi-plex Primers			
Primer	Sequence	Product Size (bp)	Lab
Pool 1:			
focG-F	5'-TGG CCA CCG GTC TTA AC -3'	385	Riordan Lab
focG-R	5'-TGC CCA TTG CTC ACT GTA AC -3'		
iutA-F	5-TCT GTT CAA TGC TGG TCT GC -3'	189	Riordan Lab
iutA-R	5'- TCC ACG CCT TCC AGC TTA CT -3'		
kpsMT-F	5'- CAT GTT CCC TCT GCA CTC AA -3'	611	Riordan Lab
kpsMT-R	5'- GCC TAA ACG CGA TCG CAT ACC -3'		
Pool 2:			
traT-F	5'- AGC GTT AGG TGC CGG TAT TA -3'	323	Riordan Lab
traT-R	5'- CGA GAA CAG GCT TCG CCT CTT C -3'		
papA-F	5'- GCA CCA TGT GGT ATT GAT GC -3'	205	Riordan Lab
papA-R	5'- GGA CAC CCG AAA AAG TCA GA -3'		
fimH-F	5- CAA AAC CTG GTC GTG GAT CT -3'	248	Riordan Lab
fimH-R	5'- CTG CTC ACA GGC GTC AAA TA -3'		
sfaD/focG-F	5'-CTC CGG AGA ACT GGG TGC ATC TTA C-3'	410	Johnson Lab
sfaD/focG-R	5'-CGG AGG AGT AAT TAC AAA CCT GGC A -3'		
Single Plex:			
hlyA-F	5'-GAT ACC GGA ACT AAA GCT GCA GC '3'	1111	Riordan Lab
hlyA-R	5'-CGT AGA TAT TGG CTG AGC CGG C-3'		

Type Specific PCR

EAEC-PCR

Type-specific PCR was performed on ST34s, as they clustered with known EAEC clinical

isolates in phylogenetic analysis. EAEC screening was performed via the specific EcoRI-PstI fragment on the pAA plasmid. Primers used were pCVD432/start (5'-CTGGCG AAA GAC TGT ATC AT-3') and pCVD432/stop (5'-CAA TGT ATAGAA ATC CGC TGT T-3') for 30 cycles as described by Schmidt et al (37). Positive and negative controls were completed with each cycle, the Giron Lab EAEC042 strain and the Johnson Lab JJ055 respectively.

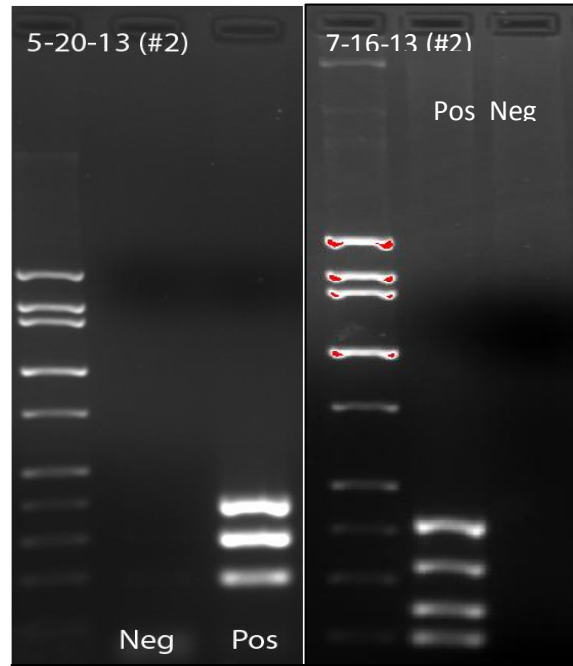


Figure 8 Multi-plex PCR Pools
 Electrophoresis gel for Pool 1 (left) and Pool 2 (right). JJ055 DNA template was used as a negative control. CFT073 DNA template was the positive control for Pool 1 of iutA (189bp), focG (385bp) and kpsMT (611bp). The positive control used for Pool 2, papA (205bp), fimH (248bp), traT (323bp) and sfa (410bp), was a 50/50 mix of CFT073 and EAEC042 DNA template.

Stx-PCR

ST171s were tested for stx2A and upon banding, stx2ab for further potential shiga toxin production using TW06296 and K12 for positive and negative controls respectively. Primers were created using stx2A530/start (5'- CAG AGC AGT TCT GCG TTT G- 3') and

stx2A704/stop (5' - CGA TAC TCC GGA AGC ACA TT-3').

Blood Assay

Isolates MCC1- MCC116 were sequentially streaked to 5% Sheep Blood Agar Plates and placed in ambient air incubators at 37°C for 18 hours. Analysis of colonies was defined as production of no hemolysis, partial hemolysis or complete hemolysis. A white light was placed under each plate for clarity. CFT073 and JJ055 were positive and negative controls respectively.

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CHAPTER THREE:

CONCLUDING REMARKS

These phlogenetic striations from normal ExPEC *E. coli* pathogens warrant further investigation into the interworking of the *E. coli* types infecting cancer patients. Next generation whole genome sequencing and Southern blot hybridization should be employed to further investigate important clonal groups/sequence types in the patient population at MCC and other cancer centers to discover mechanisms behind novel virulence, antibiotic resistance and other factors promoting this fitness niche.

Whole genome sequencing will enable deciphering of specific mutations which may currently be undocumented due to constant fluctuation in pathogenic *E. coli*. If upon further confirmation, this predominating sequence type is ST131, previous whole genome sequencing studies of clinical *E. coli* ST131 suggest incredible diversity of three clades within this particular sequence type due to increased recombination events(1). ST131 has been associated with the spread of extended-spectrum beta-lactamase (ESBL) clones. Additionally, fluoroquinolone, aminoglycoside, trimethoprim-sulfamethoxazole and carbapenem resistance have all been documented in ST131 isolates (2, 3) .

Many studies support a dissimilar array of virulence genes within the ST131 group. This disparity in virulence genes results in unknown virulence potential in each individual case.

Continuing whole genome sequencing may expose specific sequence trends within the genome to allow for better detection and assessment of pathogenicity. Previous sequencing provides insight into the fluoroquinolone resistance found in some ST131 strains showing point mutations in *gyrA* and *parC* genes (4). Whole genome sequencing will help to determine if similar mutations are responsible for other types of antibiotic resistance and virulence. This data may also be useful in detection of amino acid changes which impact cell function.

The unpredictable pathogenicity of ST131 strains in the clinical setting presents a critical need to better understand these types of *E. coli* for assisting in prevention and treatment of infections. The success of this pathogen specifically in cancer patients, where the mortality and morbidity are even greater than the normal population, should merit whole genome sequencing in order to describe the intricacies of this emerging pathogen.

Furthermore, collection of stool specimens pre-infection and post would be helpful in continued studies to assess if the cancer patient gut harbors specific disease causing *E.coli* that may not be as common or problematic in healthy individuals. Surveillance and selective digestive decolonization may be necessary to thwart such drug resistant *E.coli* in cases of drug resistant colonization to prevent infections in cancer patients and prevent community spread.

Finally, key databases with two different MLST methods may play a part in the dissimilarities seen in sequence types. A comprehensive comparison of *E.coli* strains for both MLST methods from the Achtman and Manning labs may help to decipher confusion of varying sequence types.

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APPENDIX:

IRB LETTER OF APPROVAL



DIVISION OF RESEARCH INTEGRITY AND COMPLIANCE
Institutional Review Boards, FWA No. 00001669
12901 Bruce B. Downs Blvd., MDC035 • Tampa, FL 33612-4799
(813) 974-5638 • FAX (813) 974-5618

September 28, 2010

Dr. James T. Riordan
Cellular, Molecular & Micro-Biology
4202 E Fowler Ave, BSF 218
Tampa, FL 33620

RE: **Expedited Approval** for Initial Review
IRB#: Pro00000160
Title: Genotypic Diversity and Antibiotic Susceptibility of Escherichia coli recovered from
Cancer Patients at H. Lee Moffitt Cancer Center

Dear Dr. Riordan:

On 9/28/2010 the Institutional Review Board (IRB) reviewed and **APPROVED** the above referenced protocol. Please note that your approval for this study will expire on 9/28/2011.

Approved Items:

Protocol Document(s):

[Study protocol \(Riordan_Greene\)_final.docx](#)

8/11/2010 2:34 PM 0.01

It was the determination of the IRB that your study qualified for expedited review which includes activities that (1) present no more than minimal risk to human subjects, and (2) involve only procedures listed in one or more of the categories outlined below. The IRB may review research through the expedited review procedure authorized by 45CFR46.110 and 21 CFR 56.110. The research proposed in this study is categorized under the following expedited review category:

(5) Research involving materials (data, documents, records, or specimens) that have been collected, or will be collected solely for nonresearch purposes (such as medical treatment or diagnosis).

Your study qualifies for a waiver of the requirements for the documentation of informed consent as outlined in the federal regulations at 45CFR46.116 (d) which states that an IRB may approve a consent procedure which does not include, or which alters, some or all of the elements of informed consent, or waive the requirements to obtain informed consent provided the IRB finds and documents that (1) the research involves no more than minimal risk to the subjects; (2) the waiver or alteration will not adversely affect the rights and welfare of the subjects; (3) the research could not practicably be carried out without the waiver or alteration; and (4) whenever appropriate, the subjects will be provided with additional pertinent information after participation.

Your study qualifies for a waiver of the requirement for signed authorization as outlined in the HIPAA Privacy Rule regulations at 45 CFR 164.512(i) which states that an IRB may approve a waiver or alteration of the authorization requirement provided that the following criteria are met (1) the PHI use or disclosure involves no more than a minimal risk to the privacy of individuals; (2) the research could not practicably be conducted without the requested waiver or alteration; and (3) the research could not practicably be conducted without access to and use of the PHI.

As the principal investigator of this study, it is your responsibility to conduct this study in accordance with IRB policies and procedures and as approved by the IRB. Any changes to the approved research must be submitted to the IRB for review and approval by an amendment.

We appreciate your dedication to the ethical conduct of human subject research at the University of South Florida and your continued commitment to human research protections. If you have any questions regarding this matter, please call 813-974-5638.

Sincerely,



Barry Bercu, MD, Chairperson
USF Institutional Review Board