National Journal of Biological Sciences

DOI: https://doi.org/10.37605/njbs.v5i1/6

Received: 26th January, 2024

Revised: 31st April, 2024

Accepted: 30th May, 2024

Published: 27th June, 2024

RESEARCH PAPER

TITLE

MOLECULAR ANALYSIS OF THE *CLOSTRIDIUM DIFFICILE* VIRULENCE GENES IN COLORECTAL CANCER PATIENTS

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MOLECULAR ANALYSIS OF THE *CLOSTRIDIUM DIFFICILE* VIRULENCE GENES IN COLORECTAL CANCER PATIENTS

ABSTRACT

Clostridium difficile (*C. difficile*) infections in colorectal cancer patients are a significant concern due to their potential exacerbation of existing gastrointestinal abnormalities. Excessive antibiotic use heightens the risk of *C. difficile* infections, particularly in cancer patients. This study aimed to assess the prevalence of *C. difficile* and its toxins in colorectal cancer patients undergoing antibiotic therapy and chemotherapy. A total of 50 patients with antibiotic-associated diarrhea and receiving chemotherapy were enrolled. Samples were analyzed using the immuno-chromatography test (ICT) and quantitative PCR. Additionally, the Cepheid Gene Xpert kit was employed to detect binary toxins A and B. Results revealed that 6 out of 50 patients (12%) tested positive for *C. difficile* and its toxins via ICT, while only 3 patients (6%) were positive through PCR. Moreover, 2 patients (4%) were found to be positive for toxin B using the Gene Xpert technique. The study's findings suggest that the prevalent C. difficile strains in this population are not highly virulent. This observation may be attributed to the early sampling strategy, conducted within the first week of initiating antibiotic therapy, possibly before the development of virulent strains. A limitation of the study was the short duration of hospital stays for most patients, necessitating early sampling. These findings underscore the need for continued monitoring and targeted interventions to mitigate *C. difficile* infections in colorectal cancer patients undergoing antibiotic treatment and chemotherapy.

KEY WORDS:

Gene Xpert PCR, C.difficile, CDI, Immuno-chromatography, Binary toxins.

1. INTRODUCTION

Clostridium difficile (C. difficile) is a gram-positive, anaerobic bacterium known for its ability to cause gastrointestinal infections, particularly diarrhea, which can range from mild to severe and life-threatening (Abt *et al* ., 2016; Isidro *et al* ., 2017). The bacterium produces toxins that damage the lining of the intestines, leading to inflammation and symptoms such as abdominal pain, fever, and diarrhea. C. difficile infection (CDI) has become a significant healthcare-associated infection, especially with the emergence of hypervirulent strains like ribotype 027 (Czepiel *et al* ., 2019). These strains are associated with increased severity of disease, higher rates of recurrence, and higher mortality rates. The risk factors of CDI include the exposure to higher generation of antibiotics, prolonged hospitalization and advancing age. Several risk factors predispose individuals to CDI. Some of the most significant risk factors are the use of antibiotics, which disrupt the normal balance of gut micro biota, along with Prolonged hospitalization and Advanced age allowing *C. difficile* to proliferate (Galdys *et al* ., 2014).

Cancer patients are particularly vulnerable to CDI due to multiple factors. Chemotherapeutic agents used in cancer

treatment can suppress the immune system, making patients more susceptible to infections, including CDI. For that reason, higher risk for CDI is reported in cancer patients as compared to normal individuals (Diorio *et al* ., 2018). The prevalence of *Clostridium difficile*is has been reported also higher in patients of the Intense care unit (ICU) and in kidney failure patients (Fang *et al* ., 2017; Hui *et al* ., 2018; Jain *et al* ., 2016). Mortality rates from CDI vary depending on the age group affected. While adults generally experience lower mortality rates compared to children, CDI-related deaths are still a significant concern, particularly among vulnerable populations (Huang *et al* ., 2009; Hung *et al* ., 2015). One of the challenges in controlling CDI is the presence of asymptomatic carriers who harbor C. difficile without exhibiting symptoms. These individuals can serve as reservoirs for the bacterium, contributing to its transmission within healthcare settings. Hospitalized patients, healthcare workers, and even visitors can unknowingly carry and spread C. difficile, leading to outbreaks and increased CDI incidence rates (Galdys *et al* ., 2014). Originally considered a harmless commensal of the gut microbiota, *C. difficile* has evolved into a significant nosocomial pathogen responsible for a considerable proportion of healthcare-associated infections (Moore, 2018). In Developed countries, the *C. difficile* is the major cause of morbidity and mortality in hospitalized community due to antibiotic-associated diarrhea which represents a major healthcare-associated problem (Dingle *et al* ., 2023). The bacterium was first isolated from healthy neonates' stools in 1935 and initially named Bacillus difficilis due to the challenges in its isolation and culturing (Noor & Krilov, 2018). Subsequent research led to its reassignment to the genus Clostridium, but early studies paid little attention to its clinical significance (Pettit, 2012).

C. difficile is a genetically diverse species with a highly dynamic and mosaic genome. Currently there are about 280 different *C. difficile* PCR ribotypes, considerably based on genome structure. A phylogenetic tree based on whole genome sequences which illustrate the relationship between *C. difficile* ribotypes is shown in Figure 1. Whilst certain ribotypes can dominate at given points in time (such as during an outbreak), ribotype prevalence tends to fluctuate both temporally and geographically (Kumar *et al* ., 2019). The transmission dynamics of C. difficile involve both endogenous and exogenous sources. Endogenous transmission occurs when disruption of the gut microbiota allows C. difficile to proliferate and cause infection (Johnson, 2015). Exogenous transmission, on the other hand, involves acquisition of the bacterium from external sources, such as contaminated surfaces or healthcare workers' hands. Factors such as host susceptibility, antimicrobial exposure, and environmental contamination play crucial roles in determining the likelihood of transmission and subsequent disease development (Johnson, 2015). Commonly there are more than 500 types of micro-organism in the colon (Principi *et al* ., 2020). A gram of feces having more than 10^{12} microbiots which enhance the resistance in colonization and inhibition of *C. difficile.* Lactobacilli and group-D enterococci show most antagonistic activity (Arrieta-Ortiz *et al* ., 2021). Anti-

microbial agents reduces micro-organisms which marks for *C. difficile* strain to colonize (Etifa, 2021). Due to C. difficile infection significant types of intestinal microflora are having risk. The protecting Bacteroides are to be influenced with the guide of anti-toxins, that permits the development of C. difficile (Serwecińska, 2020).

There are more than 400 strains of *C difficile (Krutova et al ., 2018)*. This infection may transmit via fecal-oral routs (De Graaf *et al* ., 2017) and *C. difficile* multiplies in the colon(Anonye *et al* ., 2019). Toxincreating strains cause pathogenicity. Toxins are endocytosed through colonic epithelial cells and harm the actin cytoskeleton, causing cell function (Chandrasekaran & Lacy, 2017; Jaber *et al* ., 2008). There are two toxins that together are typically required for *C. difficile* infection. Toxin A disturbs colonic mucosal cell, epithelial integrity also harms villous tips(Chandrasekaran & Lacy, 2017). Toxin B enters the cell via endocytosis and initiates apoptosis. Toxin B is more potent in its cytotoxic effect than Toxin A. The two animate monocytes and macrophages, which communicate interleukin 8, and carrying tissue invasion on neutrophils (Jain & Jain, 2023).

Most *C. difficile* infections are acquired nosocomial, and the reported ratios of the pathogenicity are noticeable within the healthcare setting (Marra *et al* ., 2020). Multiple reasons including high ratio of immuno-compromised patients and use of substantial antibiotics are involved in *C. difficile* infections (Lin *et al* ., 2018). The healthcare organization offers an environment where excessive deliberations on highly infectious spores are treated. Most of the patients with *C. difficile* infections are asymptomatic, though it has the capability of causing diseases (Howe, 2020). it was observed that only exposure is not responsible for causing diseases; whereas several factors, including the environment are involved in producing symptomatic *C. difficile* infections (Czepiel *et al* ., 2019). It has been assumed that triplet of events is needed to provoke symptomatic disease: 1.) Heightened susceptibility to infection. 2.) Exposure to and acquisition of a virulent strain of the pathogen and 3) Successful colonization and toxin production (McLure, 2019).

The protective role of the host microbiota against C. difficile colonization is significant, with disturbances in commensal microbiota being a leading cause of symptomatic CDI (Hunault, 2023). The gut microbiota consists of a complex ecosystem of bacteria that compete for resources and create a hostile environment for pathogens like C. difficile. Disruption of this balance, often induced by antibiotic therapy, can allow C. difficile to proliferate and cause disease (Hunault, 2023). Such as > 90% of symptomatic cases unfold during or not long after antimicrobial treatment (Regenbogen *et al* ., 2020). But the majority of mature patients taking anti-toxin treatment in yearly, which symbolize CDAI cases with a high proportion (Nale *et al* ., 2022). Where the "colonization barrier" are lost and bacterial ability leads to *C. difficile*-grew either exogenously or endogenously can multiply (Buckley *et al* ., 2021). So there are no resulting contaminations are estimated simply upsetting challenges of anti-microbial treatment, but instead as serious, hazardous, financial and restorative weights (Dhungel,

2020).Practically a wide range of anti-infective agents can possibly stimulate *C. difficile* disease. thusly the anti-microbial period (around 1970s) typically are carried with countless CDAIs cases (Bolotsky *et al* ., 2021). So none the less, the propensity to encourage malady is, as it were, subject to the class of anti-infection agents utilized for treatment. But the most serious hazard is frequently described to anti-infective agents with a significant anaerobic range of movement (Bolotsky *et al* ., 2021). While the expansive range of anti-infective agents, including penicillin's, cephalosporin's and most broadly clindamycin, which were utilized predominantly in 1970s (Devi *et al* ., 2019). But the recent ongoing *C. difficile* outbreak happened due to fluoroquinolone, which were essentially considered asacceptable because of uncertain movement against the anaerobic segment of the microbiota (Bainum *et al* ., 2023).

Although antibiotic treatment is considered as the major source of CDAI. While it is documented that medications or gastrointestinal procedures, including surgical stress and stool softeners can also initiate the disease (Georgescu *et al* ., 2018). while the patients with compromised immunity like cancer patients having severe underlying illness are mostly facing CDAI according to recent studies (Marra *et al* ., 2020). But CDAI associated Nephrology patients having advance chronic kidney failure were reported with high mortality (Ramesh & Yee, 2019). While those individuals who's undergoing bone marrow transplantation with various types of cancer with 29% in 180 days (Weber *et al* ., 2020). Generally, prolonged hospitalization carries an increased risk of disease, given that this may reflect increased exposure to a *C. difficile*-contaminated environment(Stuart *et al* ., 2019).

2. MATERIALS AND METHODS

2.1 Study Design

This is a cross-sectional study of the *Clostridium difficile* virulence genes in colorectal cancer patients. The simples were diagnosed in the laboratories of the King Abdullah Hospital Mansehra.

2.3 Sampling Collection

The samples were collected randomly through the Non-Probability Convenient sampling technique. The study sample included both male and female colorectal cancer patients. A total of 50 diarrheal feces specimens were taken from colorectal malignant growth patients of King Abdullah Hospital Mansehra in this examination. From those patients having a history of inflammatory bowel diseases and non-cancerous, were excluded from the study. The sample size was calculated according to the WHO calculator.

2.4 Sample size

The total sample was 50, as calculated by the World Health Organization sample size calculator based on the prevalence of the disease

$$n = \frac{z^2 (p \times q)}{e^2} = 50$$

Where n = Sample size

Z =Z score from table (1.96) at confidence interval of 95%

P =Disease prevalence from literature = 4%

q =1-p

e =Margin of the error (5%=5/100=0.05)

So, we have n=e $(1.96)^2$ (4 x 0.17) / (0.05)

2.5 Detection of Toxin A and B by Qualitative immunoassay

The colored chromatographic qualitative immunoassay was used for the determination of Toxin A and B in stool samples, using the CERTEST *Clostridium difficile* Toxin A+B kit (CERTest BIOTECH, Zaragoza, Spain). The strip consists of nitrocellulose membrane having pre-coated mouse monoclonal antibodies on the test line (T) against Toxin A-specific protein, and with rabbit polyclonal antibodies on the control line (C) of a specific protein of *C. difficile.* The tests were proceeding out step by step, as explained below by using the manufacturer's guidelines.The separate collection, vials were used for each sample containing a stick, through which sufficient homogenized fecal samples, were then introduced into the stool collection tube. Then tubes were vortexed for good sample dispersion. Into the strip, 04 drops of the homogenized and dispensed, and the result will be ready after 10 minutes. In the kit, a green-colored line indicates the control test, and the Redline revealed the positive test result.

Molecular identification of the virulence gene by PCR

Molecular detection of the virulence genes of *Clostridium difficile*was carried out in three step as explained below:

- DNA Extraction
- DNA Amplification
- Gel Electrophoresis and Visualization

DNA Extraction

DNA extraction was performed by using Favor Prep ^{TM} stool DNA isolation mini kit (Favorgen Biotech Crop, Taiwan).The following extraction steps were followed using manufacture guidelines are:

Amplification of DNA

The detection of virulence genes, toxin A and B was done through the PCR technique. For primer sequences were given to Gene link, and primers were commercially synthesized and used for PCR amplification. Amplification of DNA was done, by using specific primers of toxin A and B as given in Table: 2

Table 2.2 Specific primers used for amplification of toxin A and B genes

S.No	Target Gene	Primers	Primer Sequences (5' – 3')	Amplicon Size (bp)	References
01	Toxin A	Toxin A F Toxin A R	GAACCTGGAAAAGGTGATG AGGATTATTTACTGGACCATTTG	233 – 680bp	(Indra *et al* ., 2009).
02	Toxin B	Toxin B F Toxin B R	CTTAATGCAAGTAAATACTGAG AACGGATCTCTTGCTTCAGTC	233 – 680bp	(Indra *et al* ., 2009).

Gel Electrophoresis and Visualization

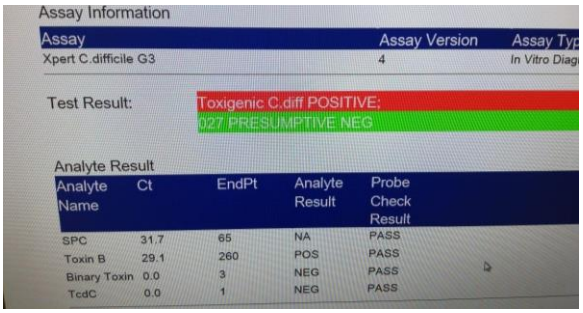
After completion of the PCR reaction, the PCR products were run on an agarose gel to observe the amplification. About 1.5 gm agarose was added to 100 ml of 1x TBE buffer and was heated in a microwave oven till the agarose was dissolved completely. The solution was cooled down and then ethidium bromide was added at 5 µl/ 100 ml of gel and mixed thoroughly. The gel tray was placed in a gel caster. Then the gel mixture poured into the tray, and coombs were placed appropriately in the tray so that a complete row of wells was formed. The gel could solidify at room temperature for 20 – 30 minutes. After the gel was solidified the coombs were removed. And the gel in the tray was transferred into a horizontal electrophoresis tank. TBE buffer (1x) was poured into the tank, so that the gel was completely submerged.

Therefore, the DNA size marker of 1000bp (Fermentas, USA) was loaded in the first well. And the samples were prepared by mixing 2 µl of 6x loading dye with 8 µl of final PCR product on a complete laboratory sealing film. The mixture was loaded into the submerged wells, and the gel was run at 120V for 40 min.When the DNA samples had migrated to an appropriate distance through the gel, the current will be turned off, and the gel will be observed under UV transilluminator. Due to the presence of ethidium bromide in the gel, the amplified products of DNA were visible as bright bands. Because of ethidium bromide intercalates between the bases of DNA and fluoresces under UV light. The gel was photographed by a digital camera. Thus the bands were analyzed in comparison with a DNA marker. Then the data was transferred to the computer for further documentation and analysis.

Cepheid's GeneXpert*C.difficile*/Epi PCR

The Gene Xpert*C.difficile*/Epi is a multiplex real-time PCR assay that detects the

virulence toxin B, the binary toxin, and tcdC gene deletion. The DNA extraction, amplification, and the targeted genes detection steps took place in different chambers of self-contained, single-use cartridge for the detection of *C.difficile*targetedtoxin genes, which contain all the necessary reagents.



Xpert *C. difficile* Assay includes reagents for the detection of Toxin producing *C. difficile* and Toxin producing *C. difficile,* presumptive 027/NAP1/BI respectively as well as the Sample –Processing Control (SPC). The SPC is present to control for adequate processing of the target bacteria and to monitor the presence of inhibitors in the PCR reaction. The Probe Check Control (PCC) verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability. The primers and probes in the Xpert *C. difficile* assay detect sequences in the genes for Toxin B (*tcdB*), Binary Toxin (*cdt*), and *tcdC* deletion 117 (*tcd*C117).

RESULTS

Interpretation of the Qualitative Immunoassay Technique

The intensity of the red-colored band in the result line will vary depending on the concentration of the antigen in the specimen. There were a total of ten samples. Out of which, only six were tested positive to the antigen against Toxin A and B. Results are given below:

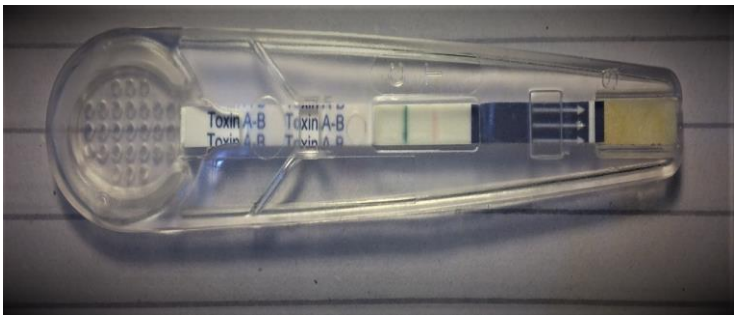


Figure: 3.1Showing positive result against *C.difficile*virulence gene Toxin A and B.

Quantification of Extracted Sample

The extracted DNA samples, then quantified by using Nanodrop (courtesy of the Forman Christian College Biotechnology Laboratory) method. Some of the results are given below:

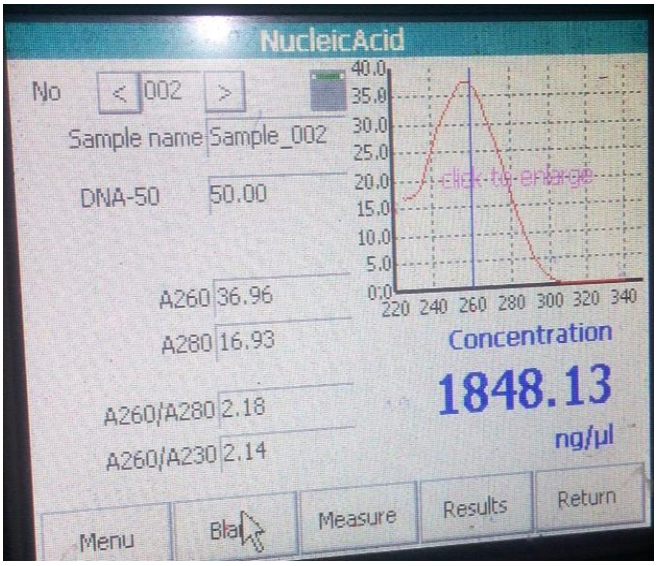


Figure: 3.2.Nanodrop Quantification curve shows Conc. Of DNA in ng/ul (Sample 1)

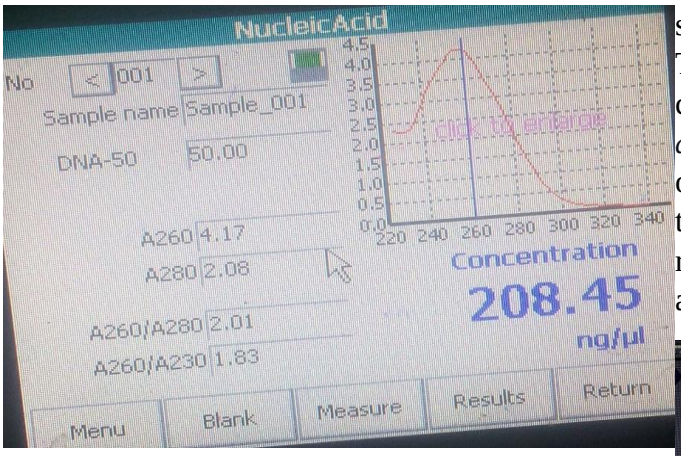


Figure: 3.3.Nanodrop Quantification curve shows Conc. Of DNA in ng/ul (Sample 2)

1.3.3 Interpretation of PCR Result

A total of fourteen isolates were subjected to PCR. Out of fourteen, the three isolates showband of size 200 – 680 bp on a gel, This suggests the presence of enzymatic domain of the Toxin A and B whose size ranges between 0-<500bp. The amplicon bands are shown in the figure below:

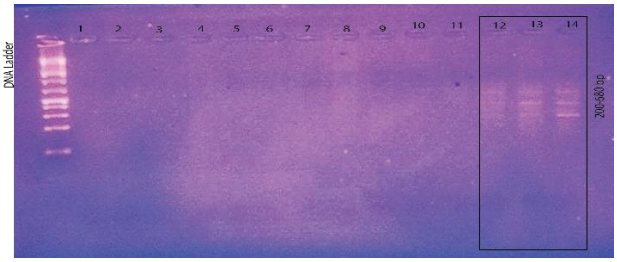


Figure: 3.4 Toxin A and B amplified by PCR process. Gel showing PCR amplification of binary toxin genes. In this figure M is 1000 bp DNA marker (Bio-Rad). NC is negative control showing no band in well number 1, sample number 1-10 (well number 2-11) showing no band while sample 11 to 13 (in well number 12-14) showing positive result of 200 – 680 bp for binary toxin A and B.

Interpretation of Cepheid's Gene Xpert Result

The gene expert results of 26 samples show varying results, those samples. Which came to be negative to binary toxin A and B in colorectal cancer patients showed only a single curve of Sample Processing Control. That is not applicable in our study as it competes with the target amplification of *C. difficile.* Also, no positive results, were observed for the presumptive 027 (no binary toxins were found in any sample) reference results of the negative sample for Toxin A and B are shown below;

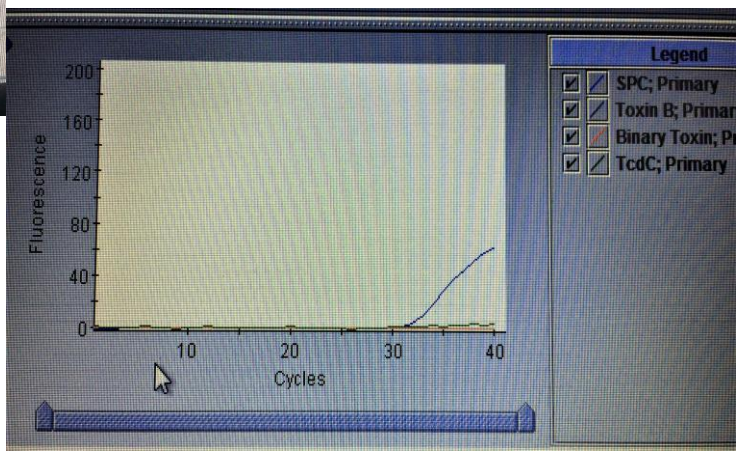


Figure: 3.5 Negative sample results for Toxin A and B. only curve is of Sample processing check that is not applicable in our study. No other significant curve can be seen.

On the other hand, from total tested samples on Cepheid PCR. Two of the patients came to be positive for the Toxin B. A valid range of Ct value was observed in the case of positive samples along with the SPC curve (that can be ignored in this study). Whereas the value of Ct produced in two cases was 29.1 with an endpoint of 260, and for the other positive sample, Ct value was 29.5 with and the endpoint of 173. Both Ct values are in the range that is valid for being positive for Toxin B, as depicted by the curves shown below, but none came to be positive for the Binary toxins.

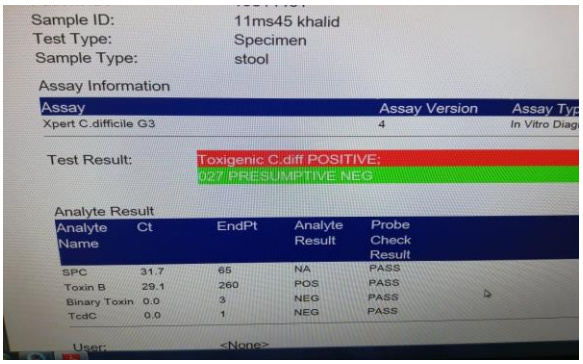


Figure: 3.6. Data of cycle threshold value for the Toxin B positive sample but negative 027 Presumptive (1st sample)

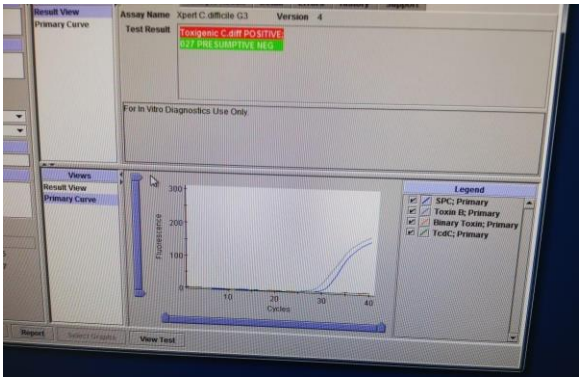


Figure: 3.7. Graph representing the Cycle threshold value curves for the Toxin B (Sample 1)

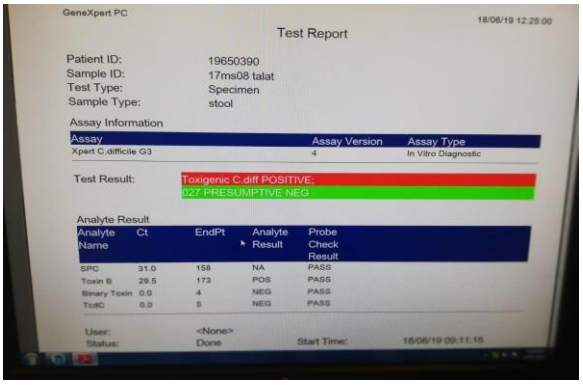


Figure: 3.8.Data of Cycle threshold value for the Toxin B positive sample but negative for 027 presumptive 2ndsamples).

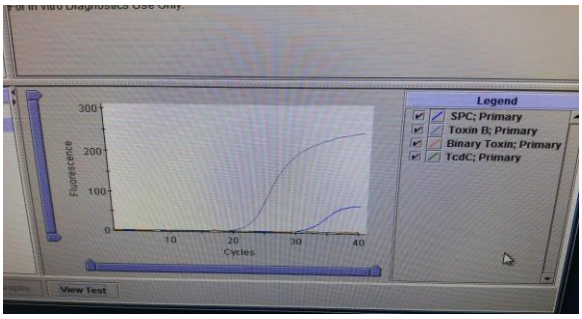


Figure: 3.9. Graph representing the Cycle threshold value curves for the Toxin B (Sample 2)

Frequency of *C.difficile* Virulence Genes

Being the most crowded city, Mansehra was found to have a majority of the chronic antibiotic user. The overall study showed that only about 20 percent population with colorectal cancer was found positive for the virulence genes of *C.difficile*. The remaining 80 percent came to be negative, for the same as depicted from the graph below;

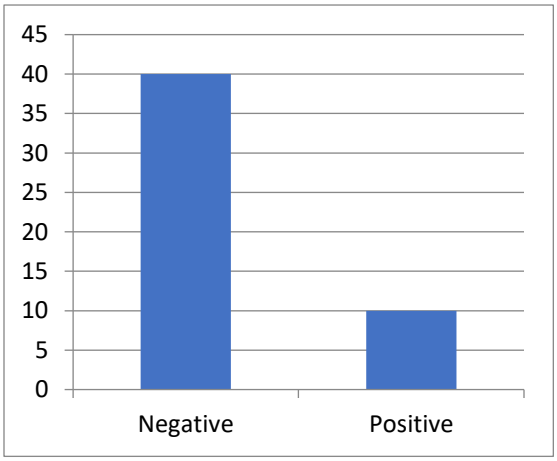


Figure: 3.10. Frequency graph of *C. difficile* positive and negative samples.

DISCUSSION

In this study, the presence of the *C. difficile*, was analyzed in the patients of colorectal cancer. And the presence of virulence genes were hypothesized to be associated with the cancer patients. By using various techniques including, strip test, PCR-gel electrophoresis, Nanodrop, and the most important, Cepheid

expert PCR performed. Out of the total, 50 ample sizes were subjected to the strip test. In which six samples gives result positive for the presence of GDH and antigen of toxin A and B. Rest of the total were analyzed for the presence of toxin domains. And PCR was run with Toxin A and B primers. In which three of them showed bands ranging in the size of the enzymatic domain of the toxins, which is one of the three-domain of the toxins, as have been discussed previously. For the Cepheid test rest of the twenty-six samples were employed, and the results showed the positive for the Toxin B. But negative for the binary toxins, tcdCetc, marked as *C.difficile* positive, and ribotype 027 presumptive negative.

The relation of binary toxins with the enhancement of the disease has been reported in various cases. Therefore, it can implement this fact in the case of colorectal cancers as well. As in a recent study it was seen that the patients that were *C. difficile* contained with binary toxin genes with toxin A and toxin B are having a high rate of mortality as compared to those with only A and B toxins in them (Bacci et al ., 2011). Also, it has been reported, that is there are binary toxins genes with the infecting *C. difficile* it will cause recurrent infection of *C. difficile* and comes as a predictive factor for recurrence (Stewart et al ., 2013).

Also, conflicts have been reported with the results. In ileal loop rabbit models, without TcdA and TcdB, binary toxins had entero-toxic response, which suggests that binary toxins having virulent nature. Whereas in the ileocolonic model of hamster, the same strains used as previous showed no symptoms of disease except colonization (Geric et al ., 2006).

In various in-vitro studies, it has been reported that binary toxins help in the induction of microtubules redistribution. And the formation of long protrusions microtubule on the surface of epithelial cells. These inductions cause the formation of a meshwork of the protrusions of microtubules on the cell surface, which helps the bacterial cells to get wrap entrapped itself, that causes increase *adherence of C. difficile on the* membrane of the intestine (Schwan et al., 2009). When, the binary toxins in the form of isogenic mutants (A- B - CDT) of the strains NAP1/027/BI, were used to inoculate the hamster's results showed that three of the total nine hamsters inoculated died with A−B−CDT+ (Kuehne et al., 2014).

Most of the assay utilized to diagnose the *C. difficile,* targets the genes of virulenceTcdA, TcdB genes. The Xpert. *difficile* promptly detects the presence of three genes, which are its target, which includes the TcdB gene, 117 deletion of tcD gene, and the TcdB gene. The development of this assay lays on the presumptive detection of the *C.difficile* infection. When the epidemic occurred due to the NAP1/BI/027 strain (McFarland, 2005; Goldenberg et al ., 2010a).

Now a day, this assay is in wide use among many laboratories of different countries for routine diagnosis of infection caused by *C.difficile*. Using antibiotics of the third-generation level. Such as cephalosporins, is reported to be the major risk factor of *C. difficile* infections (Starr et al ., 2003). Also, others like aminoglycosides, penicillin, clindamycin, are reported for causing the

environment of *C.difficile* infection (Owens Jr et al ., 2008; McFarland et al ., 2007).

C.difficile, associated colitis, is reported mostly due to the treatments, which are caused by using antibiotics. Higher surveillance and outcomes of the treatments after a cancer diagnosis is an important task to understand the features of CDI with cancers in a higher-risk environment. A prospective study should be provided to the healthcare personals to avoid or to immediately grasp the symptoms of CDI in cancer affected patients.

Colorectal cancer is among the most regularly analyzed malignancy everywhere throughout the world (Bruminhent et al ., 2014). Aside from the way of life and pre-existed comorbidities. For example, ulcerative colitis and adenomas, an adjustment in gut microbiota is viewed as a significant driving component during the carcinogenesis of CRC. As indicated by past reports, *F. nucleatum*, entero-toxigenic *B. fragilis*, and follower intrusive *E. coli* altogether found to advance the adenoma-carcinoma succession (Arthur JC 2014).

So far, the careful colonization example of *C.difficile* in CRC patients has not been resolved at this point. In an imminent report, 19% of patients were colonized with toxigenic *C. difficile* on admission to oncology (Bacci ***et al .,*** 2011). The pace of *C. difficile* colonization in kids/ adults in the hematologic ward was accounted for to be 25.6%, with 92.6% of toxigenic strains (Armin et al ., 2013). Stage T4 and LN metastasis are both high hazard factors for repeat and signs for adjuvant chemotherapy in CRC patients. Our outcomes demonstrated that CRC patients with further developed sickness (T4 or LN metastasis) who certainly need adjuvant chemotherapy. After the medical procedure will generally have a higher pace of *C. difficile* colonization. It is acknowledged that the colonization of *C. difficile* in the digestive organ is counteracted by the boundary of the gut microbiota. This shows that, the debilitating of this opposition by cancer is the major dangerous condition prompting contamination. And contrasts in colon microbiota between people with a typical colonoscopy and CRC have been accounted for (Amiot et al ., 2014; Southern et al ., 2010).

Moreover, generally longer malady course and progressively forceful treatment in patients with stage T4 and LN metastasis could bargain the assurance of gut microbiota and encourage *C. difficile* colonization. This shows changes in the organization of the gut microbiota, which may prompt the unsteadiness of homeostasis, coming about inflammation, dysplasia, and carcinogenesis (Abreu and Peek Jr, 2014, Goldenberg et al ., 2010a). Nearness and congested of *C. difficile* in CRC patients, particularly during the adjuvant chemotherapy, may create CDI and result in serious looseness of the bowels, which stops the continuous chemotherapy (Ryder et al ., 2010).

Therefore, no toxigenic strains in CDI patients were uncovered in our investigation, although the prevalence of *C. difficile* colonization in pre-operative colorectal cancer patients is there.

Besides, CDI clinical severity is commonly improved to direct inpatients in our investigation. Be that as it may, the information stays inadequate. Therefore CDI

has been common in 10% of cancer patients, the connection between *C. difficile* colonization and CDI in cancer patients still unclear. Further examinations are expected to explain the hazard variables setting off the change from *C. difficile* colonization to CDI in cancer patients.

CONCLUSION

In conclusion, the molecular characterization of *C.difficile was* extracted from the fecal sample of CRC patients. Which didn't show any toxigenic strains and only toxin B were observed by Gene XpertPCR.

A dramatic elevation of *C.difficile* nosocomial infections has been observed in the past few decades. Mostly in the cancer patients that are under treatment or had cancer surgery. The reason being the virulence effects of the strains, which are increasing in every next generation of pathogens. The emergence of the binary toxins in the genetic make-up of *C.difficile*is elevates the number of infected cases. Overall this is a preliminary study for the evaluation of toxigenic strains in the microbiota of CRC patients. Therefore, pre-operative monitoring of the CRC patients for CDI is important to avoid discontinuation of chemotherapy due to the complications resulting from CDI.

This study was aimed to check whether there is a link between the enhancements of cancer complications of CRC patients and the presence of the binary toxins. The results of our selected sample group didn't show the presence of these multiplex toxins in cancer patients. For further confirmations, the sample size needed to be increased, along with the mechanism-specific research to establish more knowledge about this relation. The role of the *C.difficile* colonization in the patients of colorectal cancer patients.

LIMITATIONS

the following study of clostridium difficile show the following limitation which could to be completed in the near future. One limitation of the study is the small sample size, which may restrict the generalizability of the findings to a larger population of colorectal cancer patients. A larger sample size would provide more robust results and strengthen the validity of the conclusions drawn from the study. The study did not include comprehensive clinical outcome data, such as disease severity, treatment outcomes, or recurrence rates. Including these data would have provided a more comprehensive understanding of the impact of C. difficile colonization on the clinical course of colorectal cancer and its treatment. Future studies should consider incorporating clinical outcome measures to better evaluate the implications of C. difficile infection in this patient population

RECOMMENDATION

One recommendation for future studies is to increase the sample size to enhance the statistical power and generalizability of the findings. A larger sample size would provide a more comprehensive understanding of the prevalence of C. difficile and its virulence genes in colorectal cancer patients. Investigating the interactions between host factors, gut microbiota composition, and C. difficile colonization could shed light on the mechanisms underlying susceptibility to *C. difficile* infection in colorectal cancer patients. Understanding these interactions may lead to the development of targeted

interventions to prevent and manage C. difficile infections in this population

AUTHORS' CONTRIBUTION (ACF):

Mr. MKK and AB Conducted the research, sampling and laboratory activity
AG TA: Contributed in conceptualization and validation of the study
NJ, NS, MS, A, A and SS SJAS: Participated in writing, and edited the methodology.
AG, NS, A, critically reviewed along with manuscript writing.All the autrhers participated in sampling and data curation and experimental work.

CONFLICT OF INTEREST : All the authors mentioned in the manuscript have no conflict in the research work and compilation.

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