PHARMACOTHERAPY IN THE TREATMENT OF *Clostridium difficile:* IMPACT ON CLINICAL PRACTICE

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NOELIA HOLGADO SÁNCHEZ

Department of Pharmacy

University of Malta

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University of Malta
L-Università ta' Malta

UNIVERSITY OF MALTA
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DECLARATION OF AUTHENTICITY FOR DOCTORAL STUDENTS

Student's I.D. /Code 134587A / 1402222

Student's Name & Surname NOELIA HOLGADO SÁNCHEZ

Course PHARM D COURSE

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DEDICATION

This thesis is dedicated to the memory of my loving grandmother Carmen Rufo Martín (1933-2017)

“Los que te quieren no te olvidan…”

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List of abbreviations

ATCC: American Type Culture Collection

BSS: Bismuth subsalicylate

CCFA: Cycloserine-cefoxitin-fructose-egg yolk agar

CDAD: *C. difficile*-associated diarrhoea

CDI: *C. difficile* infection

CDT: *C. difficile* binary toxin

CPSU: Central Procurement and Supplies Unit

EIA: Enzyme immunoassay

FDA: Food and Drug Administration

GDH: Glutamate dehydrogenase

GTP: Guanosine triphosphate

GTPases: Guanosine triphosphate hydrolases

HIV: Human immunodeficiency virus

HPV: Hydrogen Peroxide Vapour

IDU: Infectious Disease Unit

LOS: Length of stay

LTBA: Luminal toxin-binding agent

MDH: Mater Dei Hospital

MDR: Multi-drug resistant

MGEs: Mobile genetics elements

MIC: Minimal inhibitory concentration

NAP1: North American pulsed-field type 1

NGT: Nasogastric Tube

NHS: National Health System

NSO: National Statitics Office

PaLoc: Pathogenicity locus

PCR: Polymerase Chain Reaction

PMC: Pseudomembranous colitis

PPI: Proton Pump Inhibitors

REA: Restriction Endonuclease Analysis

RNA: Ribonucleic acid

SAMOC: Sir Anthony Mamo Oncology Center

SNP: Single nucleotide polymorphism

SOB: Shortness of breath

TIA: Transient ischemic attack

UTI: Urinary tract infection

WBC: Serum white blood cell

WCC: White Cell Count

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Abstract

Clostridium difficile is a pathogen accounting for 20-30% of cases of antibiotic-associated diarrhoea and is the most common cause of hospital-acquired diarrhoea. Transmission takes place by faecal-oral route. Colonization can be symptomatic or asymptomatic. Risk factors for *C. difficile* infection (CDI) include: recent or concomitant antibiotic exposure, older age, length of hospital stay, gastric acid suppression and immunosuppression.

This study aims to propose a framework for *C. difficile* culturing and antibiotic sensitivity testing with standardization of this testing procedure on the clinical setting and to identify risk factors for CDI and carriage of this infection.

Sixteen publications about *C. difficile* culturing and antibiotic sensitivity testing were reviewed and cost estimates for the materials needed to run the tests were collected. Medical records of patients with the following inclusion criteria were reviewed: over 18 years of age, inpatients at Mater Dei Hospital (MDH) or Sir Anthony Mamo Oncology Center (SAMOC) after the implementation of the "Algorithm for *Clostridium difficile* infection (CDI) investigation and results interpretation in adults" and having Glutamate Dehydrogenase antigen (GDH) positive faecal specimens. In a final phase, data available locally between 2015 and 2016 was analysed to provide an overview of the epidemiological situation of CDI.

A standard procedure for *C. difficile* culturing and antibiotic sensitivity testing in the clinical setting was proposed with a local cost of €116.30 per sample. Out of a population of 241 patients, 130 met the inclusion criteria; of whom 67 patient medical records were reviewed. Risk factors for the infection to progress to active disease were assessed.

Non-adherence to the local algorithm was detected in 13 cases. In 2015, fifty-six samples tested *C. difficile* toxin positive, compared to 111 in 2016. Recent antibiotic exposure and chronic kidney disease were identified as key factors for *C. difficile* colonization to progress to the active infection. Incidence of CDI has increased from 2015 to 2016.

According to the developed framework, it is being proposed that *C. difficile* culturing and antibiotic sensitivity testing is indicated to be performed in recurrent cases, immunocompromised patients, *C. difficile* outbreaks and for the potential establishment of a local surveillance program. The delivery of educational programs and relocation of infected patients to the Infectious Disease Unit (IDU) is recommended to improve adherence to the local algorithm. There is need for the implementation of gastric acid suppression therapy routine assessment programs to reflect on necessity for long term treatment with gastric acid suppressant drugs and a need to decrease empiric treatment with fluoroquinolones. Implementation measures to prevent contamination from *C. difficile* carriers were deemed necessary. The female gender was designated as a potential risk factor for CDI in the Maltese population and suggested for further investigations.

Keywords: *Clostridium difficile*, clinical, carriers, culturing, antibiotic sensitivity, management.

CHAPTER 1

INTRODUCTION

1.1 History

Clostridium difficile is a pathogen accounting for 20-30% of cases of antibiotic-associated diarrhoea and is the most common cause of hospital-acquired diarrhoea (Musgrave *et al,* 2011; Hood *et al,* 2014). *C. difficile* is a ubiquitous component in the gut of 2-5% of the adult population and was first reported as being part of the intestinal flora of newborn babies by Hall and O'Toole in 1935. O'Toole initiated her research in view of an increasing invasion of the intestinal tracts of new-born infants which used to occur in less than ten hours after birth and before feeding. This bacterial incursion was first observed under the microscope by Breslau in 1866 and validated by Billroth in 1874 and by Nothnagel using iodine as stain in 1881(Hill and O'Toole, 1935). It was characterized as a strict anaerobe with subterminal, non-bulging, elongated spores. *C. difficile* was initially named *Bacillus difficilis* in view of its difficulty for isolation (Hill and O'Toole, 1935; Ryan and Ray, 2004). In 1938, *B. difficilis* was re-grouped into *Clostridium* and *Clostridium difficile* was then acknowledged by the Approved List of Bacterial Names (Skerman, 1989).

A link between Pseudomembranous colitis (PMC) and CDI had not been identified when the first studies about PMC were being conducted. PMC was anatomically studied on humans by Finney in 1893 and was considered to be a complication triggered by antibiotic exposure in the 1950s. A report about a 22-year-old woman who underwent surgery for resection of a tumour in the gastric pylorus was made. The woman developed post-surgery diarrhoea and died after fifteen days. The autopsy report described a "diphtheritic membrane" in the small intestine (Finney, 1893). This description matched PMC characteristics, being identified as a complication of antibiotic treatment (Bartlett, 2009). *Staphylococcus aureus* was the suspected cause and oral vancomycin the antibiotic used (Altemeier *et al,* 1963; Hummel *et al,* 1964; Khan and Hall, 1966). The condition was

diversely named “pseudomembraneous enterocolitis”, “post-operative enterocolitis”, “antibiotic-associated enterocolitis” and “staphylococcal enterocolitis” (Khan and Hall, 1966).

C. difficile was first cultured in 1962 (Rodriguez *et al,* 2016). Studies published between 1940 and 1962 suggested that *C. difficile* was part of the normal flora and was not considered to be pathogenic (Snyder, 1940). The first antibiotic linked to antibiotic-associated diarrhoea and PMC was clindamycin in 1974 (Tedesco *et al,* 1974). In the same year, Green was conducting studies in guinea pigs about penicillin-induced death and observed cytopathic alterations in the stool specimens. He ascribed these changes to a latent virus. This was reported as the first identification of the *C. difficile* cytotoxin (Green, 1974). *C. difficile* was primarily linked to human disease and designated as the predominant bacterial cause of antibiotic-associated diarrhoea and PMC in 1977 (Ghantoji, 2010). In 1978, vancomycin was proven to be effective against toxin-producing *C. difficile* and was linked to quick clinical improvement for patients suffering from PMC (Keighley *et al,* 1978). The earliest investigation on cell cytotoxicity was carried out by Chang *et al,* in 1978. Chang *et al,* were studying antibiotic-asociated typhlitis and made evident that stool samples from hamsters with antibiotic-associated typhlitis and from individuals with PMC produced a potent cytopathic toxin that could be neutralized by *Clostridium sordellii* antitoxin (Chang *et al,* 1978). When isolates were not identified as *C. sordelli,* other clostridial species recovered from hamster stool specimens were analysed and *C. difficile* was characterized (Bartlett *et al,* 1977). Since *C. difficile* antitoxin was not attainable, the demonstration of the presence of a cytotoxin which was neutralized by the *C. sordelli* antitoxin became the standard method for detecting *C. difficile.* The first case identified by this method was in a patient with cephalothin-induced “clindamycin colitis” (Bartlett *et al,* 1978).

Polymerase Chain Reaction (PCR) has shown that *C. difficile* is associated to the *Peptostreptococcaceae* family. The relocation of *C. difficile* into a new Peptoclostridium genus with the name of *Peptoclostridium difficile* has been proposed (Yutin and Galperin, 2013).

1.2 Clinical presentation

C. difficile colonisation can be asymptomatic or symptomatic. Symptoms of *C. difficile* infection (CDI) include mild to moderate diarrhoea and life-threatening colitis. Characteristic clinical features are watery diarrhoea with 3 or more not formed stools in 24 hours (up to 15-30 depositions per day), fever (usually mild but can increase up to 40.6°C), abdominal pain with tenderness, malaise, nausea, anorexia, mucus or blood in the stool, cramping, abdominal discomfort. Laboratory results can be altered; leukocyte counts range from 10,000 to >20,000 leukocytes/mm^3 and hypoalbuminemia can be triggered by *C. difficile* protein losing enteropathy action. High C-reactive protein and increased creatinine (CRP) levels may appear in fulminant CDI (Bulusu *et al*, 2000; Bartlett 2008, Hardt *et al*, 2008; Gujja *et al*, 2009; Henrich *et al*, 2009). CDI severity can be classified as mild, moderate, severe and severe-complicated (Table 1.1).

C. difficile is a common nosocomial pathogen but cases of CDI with a community onset have been reported (Kandel *et al*, 2012; Deshpande *et al*, 2013), showing a shift in epidemiological trends. Presently, CDI is classified in three categories according to the onset of the infection (Table 1.2).

Table 1.1 Classification of CDI according to clinical features

Classification	**Symptoms**
Mild	Diarrhoea with no increase in White Cell Count (WCC).
Moderate	Intense diarrhoea and abdominal pain
Severe	Presenting with two of the following: Hypoalbuminemia (<3g/dl), serum white blood cell (WBC) count >15.000cells/mm^3, or abdominal tenderness.
Severe-Complicated	Fever >38.5°C, ileus or notable abdominal distension, signs or symptoms of colitis, mental status alterations, WBC >35.000 or <2.000 cells/mm^3, serum lactate >2.2, evidence of organ failure, admission to Intensive Care Unit, hypotension.

(Adapted from Gujja *et al*, 2009; Surawicz *et al*, 2013)

Table 1.2 Different onsets of CDI

Classification	**Characteristics**
Community onset	Patient has not been an inpatient in healthcare facility in the past 12 weeks OR symptoms show during the first 48 hours after hospitalization
Indeterminate onset	Patient has been discharged from a healthcare facility within the previous 4-12 weeks.
Health care facility onset	Patient has been discharged from a healthcare facility in the past 4 weeks or is still an inpatient.

(Adapted from Cohen *et al,* 2010)

1.3 Risk factors

The likelihood of acquiring *C. difficile* is greater when there is exposure to different factors. The length of hospital stay was shown to increase the risk for CDI by 50% after 4 weeks of stay (Gorschluter *et al,* 2001). This is due to different elements, such as: immunocompromised patients; invasive techniques leading to potential routes of infection and transfer of drug-resistant bacteria among congested hospital populations (Ducel *et al,* 2002).

Other well-known risk factors are older age (≥65 years) which results in decreased immunity and the presence of co-morbidities (Thomas *et al,* 2003; Peled *et al,* 2007). Exposure to a number of antibiotics has been identified as a risk factor for CDI. Reiterated antibiotic treatment produces dysbiosis which is a decreased diversity of the intestinal

microbiome with subsequent development of opportunistic bacteria, loss of resistance to colonization and higher release of pro-inflammatory cytokines (Brandt *et al,* 2012). This disruption promotes *C. difficile* growth which further builds up a vicious cycle of dysbiosis (Zanella Terrier *et al,* 2014).

Clindamycin was the antibiotic most commonly associated with CDI. This was reported for the first time by Tedesco *et al,* in 1974. In the 80s, cephalosporins became, to a great degree, the class of antibiotics most often involved. Broad-spectrum penicillins were designated as the second most frequently implicated agents for CDI (Keighley, 1980; Bartlett, 1981; Gilligan *et al,* 1981; Hirschhorn *et al,* 1994). Clindamycin was linked to a higher risk of CDI, whilst a number of cases were attributed to broad-spectrum penicillins and cephalosporins due to their extended use (Bartlett, 2009). Exposures to fluoroquinolones have emerged as a risk factor for CDI. Ciprofloxacin is of particular relevance since it is commonly used to treat infectious diarrhoea (Mayhew, 2011). Another class of drugs that has been reported to increase the risk for CDI are chemotherapy agents. This is attributable to the immunosuppressant nature of this class of drugs (Bilgrami *et al,* 1999; Gorschluter *et al,* 2001; Morales Chamorro *et al,* 2005). Oncology patients are at increased risk for CDI with more added risks such as length of stay and antimicrobial treatment (Chang *et al,* 2016). There is evidence that *C. difficile* has grown into the most predominant pathogen leading to bacterial diarrhoea in patients with the human immunodeficiency virus (HIV) infection. HIV patients are identified as being high risk patients in view of their underlying immunosuppression and regular antibiotic use (Sanchez *et al,* 2005). Nomura *et al,* (2009) conducted research in patients treated with immunosuppressants. Pseudomembranes are typically found in CDI as a characteristic feature, but these membranes are not present in patients using

immunosuppressive agents. Nomura *et al,* (2009) concluded that immunosuppression is associated with absence of pseudomembranes generation in CDI (Nomura *et al,* 2009).

Additional risk factors for CDI include gastrointestinal surgery and tube feeding triggered by intestinal flora perturbation with lower resistance to *C. difficile* colonization (Cohen *et al,* 2010).

Gastric acid suppression has been linked to CDI and was first reported in 1982 (Gurian *et al,* 1982). Gastric acid has been described as a bactericidal and toxin-neutralizing agent against *C. difficile* (Tennant *et al,* 2008; Cunningham *et al,* 2014). The estimated risk of CDI for patients exposed to proton pump inhibitors (PPI) varies from 1.4 to 2.75 times higher in the exposed group (Dial *et al,* 2005; Dial *et al,* 2006; Howell *et al,* 2010; Linsky *et al,* 2010; Morrinson *et al,* 2011; Kwok *et al,* 2012; Janarthanan *et al,* 2012). In 2012, the Food and Drug Administration (FDA) issued an announcement regarding this possible connection (Food and Drug Administration, 2012). It is difficult to distinguish the actual cause for CDI when several co-morbidities and risk factors are present (Loo *et al,* 2005; Pepin *et al,* 2005; Dalton *et al,* 2009). Gordon *et al,* demonstrated that the incidence of CDI is more than double when PPIs are co-administered with antibiotics, in particular fluoroquinolones and clindamycin (Gordon *et al,* 2016). Histamine-2 antagonists have been reported as risk factors for developing CDI (Hafermann *et al,* 2013; Tleyjeh *et al,* 2013). In a study by Wiedmar *et al,* gastric acid suppression therapy showed no positive association with CDI in trauma patients (Wiedmar *et al,* 2013).

Zackular *et al,* (2016) carried out a study to identify dietary zinc as a potential risk factor to develop CDI. The study was performed in mice. The mice were given different concentrations of zinc in their diets. It was concluded that zinc supplements induced a

shift in the gut flora that might lower the threshold of antibiotics which is required to increase susceptibility to CDI (Zackular *et al,* 2016).

Patients who suffer from heart failure, especially with acute exacerbation, can be more susceptible to CDI, suggesting close monitoring in these patients (Mittal *et al,* 2012). Shakov e*t al,* reported hypoalbuminemia and diabetes mellitus as being risk factors for recurrent CDI (Shakov *et al,* 2011), recommending active surveillance during admission of diabetic patients who previously had CDI. Hafermann *et al,* described higher risks for CDI in patients in the trauma-surgical intensive care unit that were administered cephalosporins as prophylaxis in the pre-operative period (Hafermann *et al,* 2013). Obesity has been suggested as a risk factor to develop CDI, although more research is required in this field (Bishara *et al,* 2013).

Chronic kidney disease has been described as an independent risk factor for CDI with unknown mechanism, being linked to increases in-hospital mortality and poor treatment responses in CDI patients (Eddi *et al,* 2010; Keddis *et al,* 2012; Kim *et al,* 2016).

1.4 Transmission and carriage

The mode of transmission for *C. difficile* is person-to-person. The bacteria are spread by the faecal-oral route in the inpatients health care setting and more recently in the community setting (Cohen *et al,* 2010; Furuya-Kanamori *et al,* 2016).

The prevalence of asymptomatic populations varies from 7-26% in the acute health care setting, to 20-50% in long-term hospital facilities where CDI can become endemic (Walker *et al,* 1993; Rivera and Woods, 2003; Riggs *et al,* 2007). A recent study in Germany by Nissle *et al.,* determined the rate of asymptomatic carriers in a geriatric unit (16.4%) which is similar to the rate in other hospital units (Nissle *et al,* 2016).

Among the carriers it is estimated that one-third to one-half will progress to symtomatic CDI, whilst the remainder are considered to remaining asymptomatic carriers (Shim *et al,* 1998; Loo *et al,* 2011). There is evidence that asymptomatic populations are a significant source for toxigenic *C. difficile.* Nosocomial CDI linked to transmission from asymptomatic carriers was reported in 1992 (Clabots *et al,* 1992). Guerrero *et al,* put forward the importance of reporting of asymptomatic carriers to implement preventive measures in view of the contribution of this population for the transmission of *C. difficile* in hospitals. In 2012, Walker *et al,* corroborated that ward-contact exchange could not justify most new CDI cases and discussed the role of the asymptomatic carriers and the spore dissemination in the transference of toxigenic *C. difficile.* In 2013, transmission of CDI from the asymptomatic population was found to be as frequent as from the symptomatic population (Eyre *et al,* 2013). There is no clear evidence about the relation between asymptomatic carriage of *C. difficile* and previous antibiotic exposure. Additional studies are required to validate this prediction pattern (Guerrero *et al,* 2013). To implement active surveillance for asymptomatic carriers, a protocol with a validated robust method must be developed (Guh and McDonald, 2016).

Rectal or perirectal swab cultures have been proposed as a diagnostic method to identify *C. difficile* carriers. Perirectal swabs have shown to cause less discomfort for patients, besides being considered safe in neutropenic patients (Freifeld *et al,* 2011). Perirectal swabs additionally render an outstanding sensitivity when compared to stool samples for the investigation of CDI. CDI patients have a greater load of *C. difficile* in stools than carriers and stool samples might be preferred for carrier's sample examination. Further studies are needed in this regard (Riggs *et al,* 2007; Kundrapu *et al,* 2012).

1.5 Toxins

Clinical isolates from patients with suspected CDI release toxin A and B or toxin B only (Johnson, 2012). Toxins are the major virulent factor for *C. difficile.* Toxins A and B are proinflammatory cytotoxic toxins that cause the disruption of the intestinal epithelial cell cytoskeleton leading to an impairment of the epithelial junctions and increased fluid in the intestinal lumen. These toxins trigger inflammatory cytokines release from mast cells, macrophages and epithelial cells resulting in additional fluid accumulation and inflammation (Bonne and Castenholz, 2001; Carter *et al,* 2010).

C. difficile toxins are encoded by *TcdA* and *TcdB* genes and form part of the 19.6 kb pathogenicity locus (PaLoc) that incorporates three regulatory genes: *TcdC, TcdR* and *TcdE.* The PaLoc are integrated in the bacterial chromosome. Mutations in these genes are accountable for toxin variant strains (Drudy *et al,* 2007). The *TcdE* gene encodes a holing-like protein that is thought to ease the release of toxins A and B from the bacterial cell since these toxins do not hold signal peptides. The signal cascade in *C. difficile* is not well described. Some studies suggest that the release of toxins is managed by the positive regulator, *TcdR,* its antagonist, *TcdC*, and the global regulator, *CodY* (Mani and Dupuy, 2001; Mani *et al,* 2002; Dineen *et al,* 2007; Matamouros *et al*, 2007). *TcdR* is described as a 22kDa protein that belongs to group 5 of the sigma 70 factor family (Helmann, 2002; Paget and Helmann, 2003; Dupuy and Matamouros, 2006). The mechanism of action of *TcdR* involves binding to the RNA polymerase holoenzyme 22 with the expression of *TcdR, TcdA, TcdB,* and very likely *TcdE* as a result. *TcdC* is suggested to work as an antisigma factor blocking the toxin generation (Braun *et al,* 1996; Matamorous *et al,* 2007). It has been proposed that variations on the *TcdC* genes may be deemed the reason for higher cytotoxicity (Spigaglia and Mastrantonio, 2002).

It has been stated that toxin B is responsible for *C. difficile* virulence (Lyras *et al*, 2009). Carter *et al*, generated isogenic *C. difficile* mutants resulting in strains lacking in the production of either toxin A or toxin B. They assessed the virulence of the different strains on the Syrian golden hamster model of infections. The results showed that toxin B is essential for disease (Carter *et al*, 2010).

1.6 New strain NAP1/BI/027

The incidence of CDI has shown a steady increase during the last 10 to 15 years (Bartlett, 2009). A hyper virulent strain named NAP1/BI/027, which was rarely detected previously, has spread with an increase in morbidity and mortality (Kuijper *et al*, 2008; Mayhew, 2011; Pant *et al*, 2011; Weber *et al*, 2013). Hospitals in the United States described an escalation in the amount of severe cases of CDI between 2001 and 2003. This strain of *C. difficile* was characterized as toxinotype III, restriction endonuclease analysis (REA) group BI, by pulsed-field gel electrophoresis as North American pulsed-field type 1 (NAP1), and later by PCR ribotyping as type 027 resulting in *C. difficile* NAP1/BI/027 strain (McDonald *et al*, 2005; Killgore *et al*, 2008).

Outbreaks of the NAP1/BI/027 strain took place in in the United States and Canada (Loo *et al*, 2005; McDonald *et al*, 2005). NAP1/B1/027 is the most common strain in North America (O'Connor *et al*, 2009). In the United Kingdom, the NAP1/B1/027 strain justified more than 40% of the cases of CDI, being frequently detected in Europe and Australia (Kuijper *et al*, 2008; Bauer *et al*, 2011; Richards *et al*, 2011).

Bauer *et al*, carrier out a study on the prevalence of the different strains of *C. difficile* in Europe. They obtained information from 395 clinical isolates, resulting in 65 PCR identifications. PCR-ribotype 027 was reported as the sixth most prevalent. The highest

prevalence was noted in the UK, followed by Ireland and Finland. PCR-ribotypes 014 and 020 remained as the most prevalent (Bauer e*t al,* 2011).

The reason for the wide expansion of the NAP1/B1/027 strain is unknown. Changes in the *TcdC* gene of the PaLoc have been suggested as being responsible for this genetic variation (Loo *et al,* 2005; McDonald *et al,* 2005; Warny *et al,* 2005; Carter *et al,* 2011). He *et al,* conducted an investigation on the genome of a global collection of *C. difficile* NAP1/B1/027 and provided robust data that suggests that the acquisition of the fluoroquinolone resistance in two separate lineages is linked to the manifestation of *C. difficile* NAP1/B1/027 (He *et al,* 2013).

This fluoroquinolone resistant strain affects affects individuals who were before classified as low risk for CDI, such as, peripartum women, children, antibiotic-naïve patients, and patients with minimun or no recent healthcare exposure (Kim *et al,* 2008). Besides the usual toxins A and B, this strain produces a third binary toxin with an undetermined pathogenic consequence (McDonald *et al,* 2005; Warny *et al,* 2005).

The *C. difficile* strain responsible for outbreaks was first reported in 1984, and isolates from 1984 to 1992 are available in the Veterans Admission Hospital *C. difficile* collection, in Hines, Illinois, the United States. This strain was detected in occasional cases of CDI throughout a 10-year period. When compared to the current isolates they show to be indistinguishable except for the fact that the previous isolates were not resistant to the modern fluoroquinolones gatifloxacin and moxifloxacin as the present isolates are (O'Connor *et al,* 2009).

C. difficile has been identified in community isolates in the past few years with *C. difficile* NAP1/B1/027 being the prevalent strain. This makes *C. difficile* responsible for community associated diarrhoea and hospital acquired diarrhoea. This shows a shift in

previous preconceptions regarding the genesis of this infection (Furuya-Kanamori *et al,* 2016).

C. difficile NAP1/B1/027 strain is known to release a third toxin named binary toxin (CDT). This toxin is formed by two subunits, member of the family of binary ADP-ribosylating toxins and functions by enhancing toxin A and toxin B toxicity (McDonald e*t al,* 2005). CDT is believed to be present in different *C. difficile* strains, and is responsible for increased mortality in patients (Gerding *et al,* 2014).

1.7 Epidemiology

In the United States, the reported incidence for CDI was 60 per 100,000 population in 2003, which is approximately double the incidence of CDI in the year 2000 (DePestel and Aronoff, 2013). In Europe, the prevalence ranges from 39% as reported in Poland to 0% as reported in Luxembourg. Between 2007 to 2008 there were 13,875 cases of *C. difficile*-associated infection reported to the Health Protection Agency in the United Kingdom (Health Protection Agency, 2011).

In 2008, the number of patients tested varied from 3 per 10 000 patient-days in Bulgaria and Romania to 141 per 10 000 patient-days in Finland (Table 1.3). In the United Kingdom before 2003, ribotype 001 accounted for 60% of cases of *C. difficile*-associated diarrhoea (CDAD). In 2008 40% of *C. difficile* isolates from 21 hospitals in South East England were ribotype 027, 21% ribotype 106 and 10% ribotype 001. The PCR-ribotype 027 is prevalent in the United Kingdom and Ireland (Bauer *et al,* 2011).

Table 1.3 Epidemiology of *C. difficile* in different countries in Europe

Country	**Toxin positive/patients tested**	**Number of patients tested per 10,000 patient/days**	**Number of participating hospitals**
Austria	53/330 (16%)	52	3
Belgium	16/283 (6%)	55	3
Bulgaria	2/9 (22%)	3	3
Croatia	22/197 (11%)	41	2
Cyprus	1/28 (4%)	34	1
Czech Republic	10/152 (7%)	17	3
Denmark	28/330 (8%)	74	3
Finland	52/351 (15%)	141	3
France	37/626 (6%)	42	4
Germany	93/602 (15%)	72	5
Greece	21/288 (9%)	60	3
Hungary	22/333 (7%)	38	3
Iceland	6/0	-	1
Ireland	38/493 (8%)	94	3
Italy	57/533 (11%)	39	5
Latvia	13/64 (20%)	10	3
Luxembourg	0/28 (0%)	49	1
Netherland	18/309 (6%)	69	3
Norway	37/241 (15%)	50	3
Poland	102/263 (39%)	45	3
Portugal	14/158 (9%)	45	2
Romania	1/11 (9%)	3	1
Slovakia	10/91 (11%)	16	2
Slovenia	24/123 (20%)	17	2
Spain	46/485 (9%)	45	5
Sweden	69/430 (16%)	74	3
Switzerland	16/150 (11%)	45	3
Turkey	4/105 (4%)	4	5
United Kingdom	164/1695 (10%)	115	6

(Adapted from Bauer *et al*, 2011)

There is a shortage of data related to the incidence of CDI, due to disagreement in epidemiological definitions and different research approaches (Shears *et al,* 2010).

There is a low reporting rate in epidemiological studies in regions outside Europe or North America. In Africa, investigations were conducted in Zimbabwe, Kenya and Nigeria, showing a reported prevalence of 8.6% (in 268 stool samples) in 2014, 56.5% (in 115 stool samples) in 2015, 43.5% in HIV-positive inpatients (in 23 stool samples between 2008 and 2009) and 14% in HIV-positive outpatients (in 71 stool samples) between 2008 and 2009 (Simango and Uladi, 2014; Rodriguez *et al,* 2016).

In Asia, the prevalence was 5.1% in Hong-Kong (in 723 stool samples) in 2008. The main strains described in Asia are PCR-ribotype 017 and 018. In Europe and America, PCR-ribotype 027 and 078 were reported (Collins *et al,* 2013).

In South America, the highest prevalence was detected in Brazil (44.9% of the patients with diarrhoea in the intensive care unit) in 2002 (Marcon *et al,* 2006; Balassiano *et al,* 2012). There is no published epidemiological data for Malta. In Malta, 56 stool samples tested positive for CDI out of the 1968 faecal specimens tested in 2015.[1] This was not official data.

1.8 Recurrence

Recurrence can be defined as total remission of CDI symptoms while on suitable therapy, followed by reappearance of diarrhoea and other symptoms after treatment is discontinued. A distinction should be made for persistent diarrhoea, described as an absence of resolution during initial therapy which requires re-evaluation (Young and McDonald, 1986; Wilcox e*t al,* 1998).

[1]Parascandalo AR, 2016, personal communication, 10th May 2016

Recurrence can be a consequence of reinfections by the strain responsible for the previous episode or with a new strain of *C. difficile.* PCR-ribotyping needs to be performed to distinguish whether the case consists of re-infection or a new infection. Studies suggest that reinfections account for the majority of recurrent cases (Barbut *et al,* 2000; Noren *et al,* 2004).

Recurrent infections are found to be frequent. Data indicates that 15-35% of patients with CDI experience a recurrence within a 12-week period of time (McFarland *et al,* 1994; McFarland *et al,* 1999; Garey *et al,* 2008; Johnson *et al,* 2009). Patients with no less than one episode of recurrent *C. difficile* have a 45 to 65% probability of further episodes (Chintalapally *et al,* 2015; Shields *et al,* 2015). The rate of recovery after an episode of recurrence drops to 40-50% for the first recurrent case and to 60% for the second recurrence (Petrella *et al,* 2005; McFarland *et al,* 2008).

A consensus definition of recurrence among different countries is not established, and no period of time between the first and the recurrent episode is officially recognized by clinical guidelines (Wiegand *et al,* 2012). Definitions for recurrence have been published. The Infectious Diseases Society of America defines recurrence as 'The presence of diarrhoea, defined as passage of three or more unformed stools in 24 or fewer consecutive hours after discontinuation of therapy' (Cohen *et al,* 2010). In the United Kingdom, there is no approved definition and several descriptions have been advised, such as 'diarrhoea after treatment completion with liquid stools remaining CDI toxin positive on repeat testing for four weeks' or 'additional diarrhoea after resolution that required another course of treatment 30 days or less after completion of treatment' (Sundram *et al,* 2009; Wilson *et al,* 2010).

The lowest reported CDI recurrence was in Germany and Switzerland (3-4%) (Fenner *et al,* 2008; Graf *et al,* 2009). Ireland showed the highest reported recurrence in 2007 with a 36% rate for first recurrence and 27% for second recurrence. The responsible strain was described as a toxin A-negative, toxin B-positive and presented a high level of resistance to the fluoroquinolones tested (Drudy *et al,* 2007). The United Kingdom and the Netherlands exhibited an elevated recurrence rate, approximately 20% in both cases between 2009 and 2010 (Debast *et al,* 2009; Wilson *et al,* 2010).

CDI has been increasing in incidence and limited data on possible risk factors for recurrence is available (Garey *et al,* 2008). A higher recurrence rate following treatment with metronidazole was described by Vardakas *et al,* when compared to prospective analysis in studies carried out during 2001-2005, in Europe and North America (Vardakas *et al,* 2012). Significantly fewer treatment failures were reported with vancomycin than with metronidazole. Kelly, 2012, stated that more than 25% of patients will have a recurrence within one to three months regardless of the treatment delivered (Kelly, 2012). There was strong evidence of absence of resistance of *C. difficile* to vancomycin and metronidazole, but new studies have reported some resistance mechanisms of this bacteria analysed by whole-genome sequencing (Pelaez *et al,* 2002; Richardson *et al,* 2015; Spigaglia, 2016).

1.9 Resistance

C. difficile isolates were disclosed as carriers of transferable genetic units, chromosomal antibacterial resistance or genetic mutations that show resistance to macrolides, lincosamides, streptogramins, tetracyclines, chloramphenicol, rifamycins and fluoroquinolones (O'Connor *et al,* 2009). Fluoroquinolones were reported to be the

prevailing cause for developing CDI (McFarland *et al*, 1990; Cartmill *et al,* 1994; Spenser, 1998).

The emergence, spread and persistence of *C. difficile* resistant strains is believed to be influenced by three main factors: Mobile genetics elements (MGEs), alteration in the antibiotic targets and multifactorial origins. MGEs are fragments of DNA that encode enzyme and other proteins involved in the migration of DNA within genomes. The alteration in the antibiotic targets is triggered by mutations in the genes that encode for the targeted proteins. Altered expression of redox-active proteins, iron metabolism and DNA repair, sessile cells showing increased resistance to metronidazole and alterated metabolic pathways involving pyruvateferrodedoxin oxidoreductase are factors from different origins involved in the *C. difficile* resistance (Lynch *et al*, 2013). The *C. difficile* genome is composed by up to 11% of MGEs providing genetic plasticity (Brouwer *et al,* 2011).

Ratnayake *et al,* described an outbreak of CDI precipitated by high-level clindamycin-resistant ribotype 106. In this case, the resistance was not interceded by the gene *ermB,* which encodes for a rRNA adenine N-6-methyltransferase. This gene was not detected by PCR in the different examined isolates during the outbreak, making the mechanism of resistance of clindamycin in this strain unknown (Ratnayake *et al,* 2011). Huang *et al,* analysed 283 strains from patients at the university-affiliated hospital in Texas and identified rifampicin resistance in 49 strains. The resistance rate was reported as 17% in this study, whilst a rate of 8% was reported in previous studies (Huang *et al,* 2013).

A metronidazole-resistant strain was studied and revealed aberrant growth in broth and elongated cell morphology. A Single Nucleotide Polymorphism (SNP) variation within

the genes involving core metabolic pathways (electron transport, iron utilization and energy production) was characterised by Lynch *et al,* in 2013 (Lynch *et al,* 2013).

Tenover *et al,* tested 316 toxigenic clinical isolates of *C. difficile* in the United States for susceptibility to metronidazole, clindamycin, moxifloxacin and rifampin. Forty-one-point five percent of the isolates showed resistance to clindamycin, 38% to moxifloxacin, 7.9% to rifampin and one case was metronidazole-resistant. Multi-drug resistance was reported in 22 of 80 *C. difficile* PCR-ribotype 027 isolates (Tenover *et al,* 2012).

Freeman *et al,* reported findings of epidemiological and antimicrobial susceptibility studies of *C. difficile* isolates in 2014. These isolates showed resistance to clindamycin (49.62%), moxifloxacin (39.99%), rifamycin (13.40%), imipenem (7.41%), chloramphenicol (3.70%), metronidazole (0.11%) and vancomycin (0.87%) (Freeman *et al,* 2014).

Resistance to vancomycin was previously described by Pelaez e*t al,* Three-point one percent of the *C. difficile* isolates were resistant to vancomycin in a studied carried out in Spain in 2002 (Pelaez *et al,* 2002).

Jamal and Rotimi also described resistant isolates to metronidazole (2.9%) in hospital-acquired diarrhoea (Jamal and Rotimi, 2016). A new metronidazole-resistant strain emerged with a high treatment failure rate as a consequence (Moura *et al,* 2013). Although resistances to metronidazole had been reported from 1997, there was an increase in the number of cases from 2008 (Richardson *et al,* 2015; Spigaglia, 2016).

C. difficile has turned into a multi-drug resistant (MDR) bacteria. Spigaglia *et al,* reported that 55% of the resistant strains were MDR. Forty nine percent of the MDR isolates showed resistance to four distinct groups of antibiotics: macrolides (erythromycin),

lincosamide (clindamycin), fluoroquinolones (moxifloxacin) and rifamycin (Spigaglia *et al*, 2011).

One case isolate was described as resistant to fidaxomicin after recurrence (Minimal Inhibitory Concentration (MIC) =16mg/L). This was not considered to be of clinical significance (Goldstain *et al,* 2012). The mechanisms of mutations for reduced susceptibility to fidaxomicin have been described *in vitro* (Leeds *et al*, 2014).

1.10 Mortality

The appearance of the new hyper virulent strain NAP1/BI/027 is responsible for the increase in mortality due to CDI in the past years. *C. difficile* mortality rate is estimated at 2% (Cohen *et al,* 2010; Jones *et al,* 2013). Reported mortality rates from (CDI) in the United States rose from 5.7 per million in 1999 to 23.7 per million in 2004. No ribotype was specified. In the United Kingdom, the number of deaths increased likewise, from 1804 in 2003 to 8324 in 2007 (Creagh, 2008). Karas *et al,* carried out a meta-analysis of 27 articles about *C. difficile* mortality for the period of January 1980 to March 2010. The results were heterogeneous in terms of definitions, patient groups, type and quality of study, information collected and accessible data. Thirty-day mortality rate measures death occurring within thirty days of a defined event. This statistic was selected as the outcome with a general high mortality, average of 5.99% among the different publications (Karas *et al,* 2010). The review suggested an increase in mortality, showing a 2.5-fold increase after 2000. The greatest attributed reason was the emergence of a hyper virulent strain, NAP1/BI/027 (Karas *et al*, 2010).

In-hospital mortality measures death occurring during hospital stay. In-hospital and 30-day mortality were selected as outcomes in the review by Wiegand *et al*. This study reviewed 31 articles across 10 European countries, including Austria, Denmark, Finland,

France, Spain, Germany, Ireland, Luxembourg, Switzerland, UK and the Netherlands. The 30-day mortality fluctuated from 6.8% in Ireland to 42% in the UK (Drudy *et al,* 2007; Gulihar *et al,* 2009). The high mortality in the United Kingdom stands out, 35% in-hospital mortality in 221 cases of CDI during an outbreak was reported in 2009. Eighty-five percent of the detected strains were ribotype 027, linked to increased complications and mortailty (Freeman *et al,* 2010). A CDI in-hospital mortality rate of 44% was noted in Austria, 27% in France, 15% in Luxembourg and 14% in Spain (Coignard *et al,* 2007; Vicente *et al,* 2008; Wiegand *et al,* 2012). In Germany, a 52.2% general mortality in patients with CDI described as severe was disclosed in 2010 by Eckmanns *et al,* (Eckmans *et al,* 2010).

CDI reporting became obligatory in the United Kingdom in 2004 for patients aged over 65 years and for all patients over 2 years in 2007. The United Kingdom has the widest CDI reporting scheme in Europe (Department of Health and Protection Agency, 2012). Before 2007 the reported number of deaths in England and Wales was more elevated in men than women; after 2007, the mortality rate was greater in women than men. According to the Office for National Statistics of England and Wales, the mortality rate reached its maximum in 2009 with 461 deaths and the lastest data shows 108 deaths in Wales in 2015 (Office for National Statistics, 2015).

Due to the difficulty to identify the reason of death in patients with co-morbidities and CDI, death reported with CDI as a cause, can triger misleading interpretations of the true CDI mortality rate (Wiegand *et al,* 2012).

1.11 Economic burden

The rate of sample testing for *C. difficile* differs among countries and hospitals. Patient/days is used to calculate the rates of CDI and is defined as patient episodes of hospital identified CDI (total x 10.000 hospital CDI cases) as the numerator and number of patient days in hospital as the denominator.

In Finland, 141 patients were tested per 10,000 patient/ days; whilst Bulgaria and Romania reported 3 patients tested per 10,000 patient/days (Bauer *et al,* 2011). Data shows that CDI is underestimated by a large proportion of clinicians worldwide and there is a low level of awareness of CDI (Mavros *et al,* 2012).

CDI creates a burden for the National Health System (NHS) with increasing lengths of stay (LOS) in hospital. LOS due to CDI have shown to be more elevated than an average hospital stay. It was determined that each CDI episode was linked to a 14 days of added hospital stay in the United Kingdom and a mean of 37 days of hospital stay (Rodrigues *et al,* 2010; Wiegand *et al,* 2012).

The costs incurred by CDI are related to testing, treatment and environment and worker's disinfection. The estimated annual cost for the NHS to treat CDI is close to £75 million (National Audit Office, 2009). In Germany, a median cost of €33,840 per CDI patient was reported in 2008 (Vonberg *et al,* 2008). In Ireland, the annual incremental cost of CDI was £2691 between 1994-1995 which equals to £4577 when converted into comparable units in 2000 (Al-Eidan *et al,* 2000). Reddy *et al,* described an annual incremental cost of £126,500 due to increases in *C. difficile* toxin testing. In Finland, an incremental cost of €2300 per CDI case was described in 2009 (Agthe *et al,* 2009).

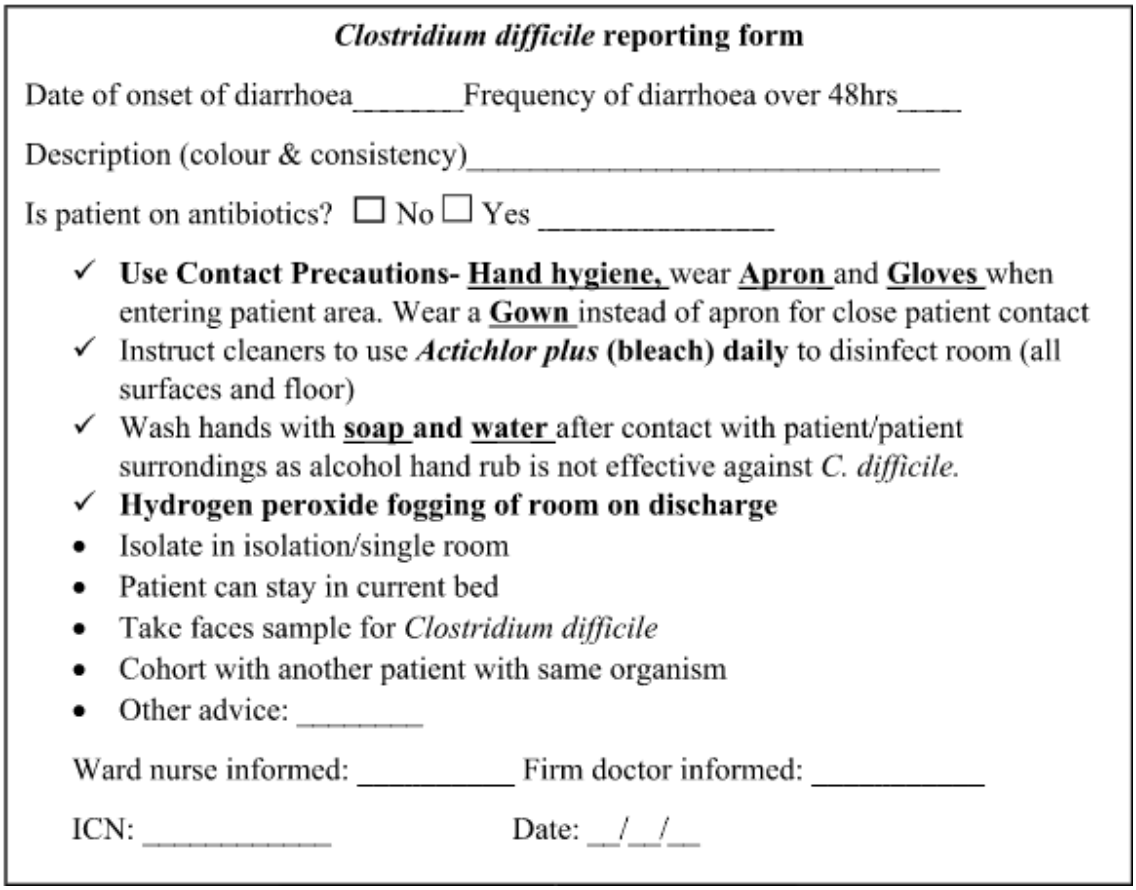
CDI is considered one of the most expensive nosocomial infections due to the high expenditures linked to prolonged hospitalization and rehospitalisation (National Institute for Health and Care Excellence, 2012). The cost attributed to CDI was €3 billion per year in the European Union, considering 500 million inhabitants (European Centre for Disease Prevention and Control, 2013). The estimated cost for CDI in the United States was between $436 million and $3.2 billion per year (O'Brien *et al*, 2007; Dubberke *et al,* 2008). Wang and Stewart compared the costs attributable to CD-colitis and non-CD-colitis. The mean cost per admission for CD colitis was over double the amount than the admissions for non-CD colitis during the years 2005-2008 (Wang and Stewart, 2011).

1.12 Contamination and disinfection

C. difficile spores can remain on inanimate surfaces up to five months (Kramer *et al,* 2006). Bedside tables, bed frames and floors are reported as the most contaminated surfaces in rooms where patients with CDI were isolated (Verity *et al,* 2001). The spores are 10 to 15 times more resistant than the vegetative bacteria to detergents (Davies *et al,* 2011). Contamination of hands of health care workers and patients is also a relevant factor for transmission of *C. difficile* (Dancer, 2009). Different methods are suggested for disinfection of the affected areas in hospital. Terminal cleaning of a mattress, bed space or ward areas after discharge, transfer or death of a patient with CDI can be effectuated by hydrogen peroxide vapour (HPV) dry ozone oxidizing technology, chlorine-releasing agent, microfibre cloths or high temperature, (180°C) over heated dry atomized steam cleaning (Polti® steam) in combination with a sanitizing solution, a hydro-alcohol solution containing sodium metasilicate and sodium carbonate, steam cleaning or clorhexidine sporicidal wipes (Doan *et al,* 2012). Efficient disinfection at the time of patient discharge lessens *C. difficile* acquirement (Otter *et al,* 2011). McCord *et al,* proved a 60% reduction in the hospital-wide CDI rate and a reduction in the exposure of patients

to *C. difficile*-contaminated areas when HPV is used for disinfection, and recommend this method for terminal disinfection of rooms vacated by patients with CDI (McCord *et al,* 2016). The department of Health and Health Protection Agency in the United Kingdom currently suggests a daily environmental cleaning of rooms of *C. difficile* patients with chlorine-containing cleaning agents, bathroom toilets of CDI patients should be cleaned with chlorine-containing cleaning agents after each use, all areas in the room should be thoroughly cleaned and curtains removed after patient is transferred, discharged or deceased. The use of vaporised hydrogen peroxide is considered to render total disinfection of the environment and equipment in single rooms or isolation wards (Department of Health and Health Protection Agency, 2012). It is recommended that healthcare providers wash their hands with soap and water before and after each contact with CDI patients or suspected infective diarrhoea. Gloves and aprons should be used in the case of physical contact with patients and gloves and apron should be dischard immediately after use. The application of alcohol hand rub should not substitute the use of the soap, although it can be applied afterwards for non-clostridial organisms (Department of Health and Health Protection Agency, 2012).

In hospitals locally, cleaners are instructed to use Actichlor plus® (bleach) daily to disinfect all surfaces and floor in the room. All persons in contact with the patient or patient surrondings are encouraged to wash their hands with soap and water. Alcohol hand rub is not effective against *C. difficile.* The room is fogged with hydrogen peroxide on patient discharge. The ward nurse and the firm doctor are required to sign aparticular form (Figure 1.1) for every reported case of CDI.



***Clostridium difficile* reporting form**

Date of onset of diarrhoea_____Frequency of diarrhoea over 48hrs____

Description (colour & consistency)_____

Is patient on antibiotics? ☐ No ☐ Yes _____

- ✓ **Use Contact Precautions- Hand hygiene,** wear **Apron** and **Gloves** when entering patient area. Wear a **Gown** instead of apron for close patient contact
- ✓ Instruct cleaners to use ***Actichlor plus* (bleach) daily** to disinfect room (all surfaces and floor)
- ✓ Wash hands with **soap and water** after contact with patient/patient surrondings as alcohol hand rub is not effective against *C. difficile.*
- ✓ **Hydrogen peroxide fogging of room on discharge**
- Isolate in isolation/single room
- Patient can stay in current bed
- Take faces sample for *Clostridium difficile*
- Cohort with another patient with same organism
- Other advice: _____

Ward nurse informed: _____ Firm doctor informed: _____

ICN: _____ Date: __/__/__

Figure 1.1 *Clostridium difficile* reporting form in Malta

1.13 Management and testing

C. difficile can be present in asymptomatic patients identified as asymptomatic carriers. CDI symptomatic patients can present severe complications including death (Bartlett, 2007). Measures are only taken for symptomatic patients. Asymptomatic carriers remain as an untreated source of transmission due to lack of treatment guidelines for asymptomatic carriers (Guh and McDonald, 2016). Current guidelines for the management of CDI do not recommend routine screening for *C. difficile* in inpatients with no symptoms or treatment of asymptomatic carriers (Surawicz *et al,* 2013).

Screening for *C. difficile* is recommended in the presence of diarrhoea and recent or current antibiotic treatment (Dellit *et al,* 2007; Cohen *et al,* 2010; Jury *et al,* 2013). British

guidelines put emphasis on the mnemonic protocol SIGHT for managing patients with suspected infectious diarrhoea (Table 1.4).

Table 1.4 British recommendation guidelines when managing patients with suspected infectious diarrhoea.

S	Suspect CDI if there is no other alternative cause of diarrhoea.
I	Isolate the patients whilst awaiting laboratory results.
G	Gloves and aprons must be worn while in contact with the patient to avoid contamination.
H	Hand disinfection after contact with the patient.
T	Test the stool for *C. difficile* toxin.

(Adapted from Department of Health and Health Protection Agency, 2012).

British guidelines recommend daily monitoring of diarrhoea for frequency and severity using the Bristol Stool Chart (Appendix 1) (Department of Health and Health Protection Agency, 2012) whilst stool charting is not mentioned in American guidelines (Cohen *et al*, 2010).

All non-clostridial antibiotics should be stopped as well as other drugs that can precipitate diarrhoea and adequate hydration must be give to patient (Department of Health and Health Protection Agency, 2012; De Silva, 2012; Surawicz *et al*, 2013).

In most clinical settings in different countries, only diarrhoeal stools are sent for *C. difficile* investigation (Cohen *et al*, 2010; De Silva, 2012; Surawicz *et al*, 2013).

Different laboratory studies can be performed such as stool culturing, enzyme immunoassays, cell cytotoxin neutralization assay, polymerase chain reaction (PCR).

1.13.1 Stool culturing

Stool culturing stands as the most sensitive test with high value in epidemiological studies. Stool culture alone is not performed on a daily basis due to its slow turnaround time (48hours) and due to the fact that only toxigenic organisms cause disease. It is significant as a confirmatory test, and as a prior step for toxigenic culture (traditional "gold standard") or antibiotic sensitivity testing (Cohen *et al,* 2010; De Silva, 2012). Stool culturing is recommended by the British guidelines for patients not responding to metronidazole or vancomycin treatment (Department of Health and Health Protection Agency, 2012).

1.13.2 Enzyme immunoassays

Enzyme immunoassays (EIAs) detect toxin A and B with a fast turn-around time (24 hours) They are moderately inexpensive, easy to perform but show an inferior sensitivity (~ 90%). EIAs are not recommended as a single screening method for *C. difficile* (Cohen *et al,* 2010; Pant *et al,* 2011; Department of Health and Health Protection Agency, 2012; Surawicz *et al,* 2013).

C. difficile releases the enzyme NAD-specific glutamate dehydrogenase (GDH) in large amounts and can be detected in stool samples. This enzyme confers resistance to H_2O_2. The detection of GDH is sensitive but not specific for CDI. It is not valid as a single method to detect the presence of toxigenic *C. difficile* (Shetty *et al,* 2011; Bignardi *et al,* 2013; Girinathan *et al,* 2014; Cheng *et al,* 2015).

The lack of sensitivity of the EIAs and GDH detection alone can be overcome by a 2-step testing algorithm. Detection of glutamate dehydrogenase followed by a toxin test to

confirm the presence of the toxin is a recommended approach for detection of toxigenic *C. difficile* by British and American guidelines (Cohen *et al,* 2010; Department of Health and Health Protection Agency, 2012).

1.13.3 Cell cytotoxin neutralization assay

In the tissue cytotoxin neutralization assay, the presence of toxin B is detected by measuring the reaction (cytopathic effect) between the faecal supernatant and a monolayer of human or other mammalian cells in culture. It has a slow turnaround (48 hours) and poor interlaboratory precision. This approach is recommended by the British guidelines as a confirmatory diagnosis (Department of Health and Health Protection Agency, 2012).

1.13.4 Polymerase Chain Reaction (PCR)

PCR has been recommended as a confirmatory test to detect toxin B gene (Galea *et al,* 2015). Further research in this field for detection of toxigenic *C. difficile* is needed due to the lack of experience as a routine testing method (Cohen *et al,* 2010).

1.14 Treatment

Treatment for CDI can be started before laboratory confirmation in those cases where the infection is likely to be present. The general approach for a first episode of CDI is metronidazole or vancomycin and daily assessments should be performed. Oral metronidazole is suggested for mild-to-moderate cases with a regimen of 400 or 500mg (according to local availability) orally three times a day for 10-14days (switch to oral vancomycin 124mg four times a day if there is no improvement after 4-6 days). Vancomycin 125mg four times a day for 10-14 days is recommended for severe CDI (Musgrave *et al,* 2011; Yeung *et al,* 2015; Feher and Mensa, 2016). These recommendations are included in the American and British guidelines for the treatment

of CDI (Cohen *et al,* 2010; Department of Health and Health Protection Agency, 2012). Vancomycin can be administered via rectum if ileum is present. For severe cases, the American guidelines suggest the administration of intravenous metronidazole as 500mg dose given 3 times a day for 10-14 days (Cohen *et al,* 2010). Sub-total colectomy with preservation of the rectum should be considered in severe cases not responding to the above described treatment according to American and British guidelines (Cohen *et al,* 2010; Department of Health and Protection Agency, 2012).

Rifampicin 300mg given orally twice a day together with a high dose of oral vancomycin (500mg for times a day) or administration of intravenous immunoglobulin 400mg/kg in one dose with possible repetition is suggested for severe cases in the algorithm for treatment of CDI in the British guidelines. Its effectiveness is not well-known (Department of Health and Health Protection Agency, 2012).

The approach for the first recurrence is the same as above, always considering the severity of the case (Yeung *et al,* 2015). For second and subsequent recurrences, vancomycin is the preferred agent (Cohen *et al,* 2010; Musgrave *et al,* 2011; Yeung *et al,* 2015). Tapered doses of vancomycin which involve progressing lowering of the vancomycin dose throughout weeks is considered for recurrent cases according to British guidelines. Tedesco *et al,* suggested this regimen in a study with 22 patients suffering from recurrent CDI. They were treated as follows: 6-week tapered regimen of vancomycin; 125mg four times a day for 1 week, followed by 125mg twice a day for 1 week, followed by 125mg daily for a week, then 125mg every other day for a week and 125mg every 3 days for 2 weeks. There were no reported recurrences within 2 months (Tedesco *et al,* 1985).

Fidaxomicin is a novel drug that was approved by the FDA in 2011. It is a narrow-spectrum macrocyclic antibiotic and is an alternative to vancomycin in the treatment of

mild-to-moderate CDI when given 200mg orally twice a day for 10 days (Musgrave *et al,* 2011; Surawicz *et al,* 2013). Fidaxomicin shows non-inferiority to vancomycin, it is effective against the NAP1/BI/027 strain and seems to cause less disruption of the anaerobic colonisation microbiota than vancomycin, hence reducing recurrence (Miller, 2010). The cost of fidaxomicin is higher than the cost of vancomycin. Nathwani *et al,* carried out a cost-effectiveness analysis of fidaxomicin versus vancomycin and concluded that fidaxomicn is cost-effective in patients with severe CDI and first CDI recurrence (Nathwani *et al,* 2014).

Probiotics can be used. They include organisms such us *Bifidobacteria* spp, *Lactobacillus* spp or *Saccharomyces boulardii* (Vincent *et al,* 2015). The effectiveness of probiotics in treating CDI is inconclusive. Some probiotics have shown to cause "colonization resistance" to *C. difficile,* by production of antimicrobials and acids which decrease the pH of the intestine. This can complicate *C. difficile* growth (Musgrave *et al,* 2011). An analysis of randomized controlled trials suggested that the administration of *S. boulardii* together with high vancomycin doses can decrease CDI recurrence (Cohen *et al,* 2010). This administration should be outweighed in immunosuppressed patients due to the risk of fungemia (Enache-Angoulvant *et al,* 2005). Meta-analyses have failed to demonstrate their efficacy in treatment and prevention of CDI (Dendukuri *et al,* 2005; Pillai and Nelson, 2008). Probiotics are not recommended in American or British guidelines, but these guidelines suggest more research in this field (Cohen *et al,* 2010; Department of Health and Protection Agency, 2012).

Faecal transplant is based on the restoration of healthy intestinal flora by replacement of current gut content with healthy faecal samples. This technique is recommended in relapsing CDI and shows a success rate of ~90%. The stool samples must be treated by adding sterile normal saline, homogenizing in a blender and filtering the suspension prior

instillation (Musgrave *et al,* 2011; Goldenberg, 2016). The patient is usually treated with vancomycin 250mg every 8 hours before transplant to decrease *C. difficile* load and omeprazole 20mg (two doses) to reduce gastric acid production and establish an adequate environment for the bacteria (Aas *et al,* 2009; Bakken *et al,* 2009). The instillation can occur via nasogastric, nasoduodenal or nasojenunal tube, retention enema or colonoscopy and it normally entails one treatment (Aas *et al,* 2009; Bakken *et al,* 2009; Li *et al,* 2016). This approach is currently implemented in guidelines for the management of relapsing CDI (Cohen *et al,* 2010; Department of Health and Health Protection Agency, 2012; Surawicz *et al,* 2013).

1.15 Other treatment options

Metronidazole and vancomycin have effectively been used against CDI for 30 years, however there is evidence of induced-resistances, disruption of healthy intestinal flora and high recurrence rates that have led to further investigation in the treatment of CDI (Lynch *et al,* 2013; Chong e*t al,* 2014).

Toxin-binding agents have been proposed as an alternative. They work by binding the toxins in the intestinal lumen and they receive the name of Luminal Toxin-Binding Agents (LTBAs). Examples of these are: colestipol, cholestyramine and tolevamer. Current data suggest that LTBAs monotherapy is inferior to vancomycin treatment, LTBAs decrease vancomycin concentrations when administered together and they are not currently recommended in the treatment of CDI (Cohen *et al,* 2010; Johnson *et al,* 2014; McCoy *et al,* 2016).

Earlier studies have reported bismuth subsalicylate (BSS) as an antimicrobial agent besides its role as an antacid. There is no evidence to support its use in the treatment of CDI and more research is needed in this field. Pitz *et al,* suggest that there is enough data

to confirm that BSS has antimicrobial properties against *C. difficile* (Musgrave *et al,* 2011; Pitz *et al,* 2015).

Rifaximin has shown optimistic results for the treatment of recurrent CDI. It is considered safe in view of the non-absorption. It has been proposed as an alternative to vancomycin in the regimen of 400-800mg daily in 2-3 divided doses for two weeks (Marchese *et al,* 2000; Mattila *et al,* 2015).

Nitazoxanide has been reported to have the same efficacy as vancomycin for initial treatment of severe CDI or for recurrent/refractory disease. Further studies are needed to confirm efficacy. The suggested dose for the treatment of severe CDI is 500mg every 12 hours (Musher *et al,* 2009; Musgrave *et al,* 2011).

Tigecycline has shown *in vitro* activity against *C. difficile.* This antibiotic is administered intravenously and reaches high concentrations in faeces. More studies are needed to determine its efficacy and therapeutic role in CDI but preliminary studies about its role are encouraging (Musgrave *et al,* 2011; Bella *et al,* 2015; Kundrapu *et al,* 2015).

Cadazoliz and linezolid have been reported as potent agents against *C. difficile in vitro.* There is no solid evidence to suggest their use in clinical practice and *in vivo* studies are required (Locher *et al,* 2014).

Ramoplanin is a glycolipodepsipeptide that has been studied for initial treatment of CDI. The Food and Drug Administration (FDA) granted the “Fast Track” status to this drug and it was approved through Phase II studies for the treatment of CDI. Ramoplanin is suggested to be orally administered in 200mg or 400mg twice a day for 10 days (Musgrave *et al,* 2011; Kraus *et al,* 2015).

Bacteriocins are protein molecules released by bacteria that are effective against related strains. Nisin is secreted by *Lactobacillus latis* and shows a narrow spectrum

antimicrobial activity for gram-negative bacteria. Synergistic inhibition of *C. difficile* with nisin-lysozyme combination has been suggested. Lisozyme works by hydrolysing peptidoglycan bonds enhancing nisin penetration towards the cell membrane where it creates pores resulting in cell death. This combination has shown promising *in vitro* results (Chai *et al*, 2015).

Thuricin CD is an antimicrobial and is classified as a bacteriocin (sactibiotic subclass). It has been *in vitro* studied as an alternative for treatment of CDI as a monotherapy or in combination with tigecycline, vancomycin, teicoplanin, rifampicin and nitazoxanide. Its efficacy was measured by assessing the ability to inhibit *C. difficile* biofilms. The efficacy of the antibiotics tigecycline, vancomycin, teicoplanin and rifampicin is enhanced when combined with Thuricin CD. Nitazoxanide efficacy is reduced by combination with Thuricin CD. These combinations are presented as alternative approaches for the management of CDI (Mathur *et al*, 2016).

LFF571 is an antimicrobial in current Phase II investigation and is classified as a semisynthetic thiopeptide. A ramdomized trial was carried out in patients with initial episode or first recurrence of CDI in order to compare oral LFF571 200mg versus oral vancomycin 125mg. LFF571 was effective and well tolerated with higher cure rates than vancomycin, (90.6% and 78.3% respectively). This study concluded that treatment with LFF571 is noninferior to vancomycin treatment (Mullane *et al*, 2016).

A recombinant subunit vaccine against CDI has been developed, using a lipidated C-terminal receptor binding domain of toxin A (rlipoA-RBD) to contain a toll-like receptor 2 agonist that is expressed in *Escherichia coli.* The vaccine was tested in mice, hamsters and rabbits exhibiting protection against CDI and suggesting further clinical trials (Huang

et al, 2015). Recently, Gupta *et al,* provided robust evidence for the use of monoclonal antibodies targeting toxin B in the prevention of recurrent CDI (Gupta *et al,* 2016).

Wilcox *et al,* conducted two double-blind, randomized, placebo-controlled, phase 3 trial to assess the efficacy of the human monoclonal antibodies actoxumab and bezlotoxumab. This study concluded that treatment with bezlotoxumab while on antibiotic treatment for primary or recurrent CDI, shows lower infection recurrency rate than placebo. Treatment with actoxumab did not show improvement in the condition (Wilcox *et al,* 2017).

1.16 Local Scenario

Faecal specimens to be tested for *C. difficile* are processed in the virology laboratory at Mater Dei Hospital (MDH). Until April 2016, toxin A & B enzyme immunoassay was the test performed at MDH in the presence of suspicion of CDI. In April 2016, the implementation of the "Algorithm for CDI investigation and results interpretation in adults" (Appendix 2) took place. This algorithm involves an initial screening test for Glutamate Dehydrogenase antigen (GDH) of *C. difficile*, followed by a confirmatory toxin test for GDH antigen positive cases. Both tests are enzyme immunoassays.

The presence of the GDH antigen and absence of the toxin implicates the carriage of non-toxigenic *C. difficile* and is reported as "CDI equivocal". In this case, patient symptoms are assessed and a new specimen is tested if the patient is symptomatic or there is a high clinical suspicion of CDI. Isolation is recommended until the patient is diarrhoea-free for 48 hours. A second test with the same result indicates colonization rather than infection with potential for transmision.

When the result is GDH antigen positive and the toxin indeterminate, the same management as for GDH antigen positive and toxin negative is advised.

When GDH antigen test is negative, toxin test is not performed and the result is issued as "CDI unlikely". In this case the treatment against CDI is not recommended.

When GDH antigen and toxin test are positive, the result is reported as "CDI likely". Patient should be isolated in a single room and Infection Control should be informed. Where possible, non-clostridial antibiotics, antimotility agents and gastric acid suppression should be stopped and treatment initiated.

Under the presence of severity markers such as suspicion of pseudomembranous colitis, toxic mega colon, ileus or presence of colonic dilation in CT scan (>6cm), WBC >15x106/l, creatinine >1.5 x baseline, temperature >38.5ºC or immunosuppression, vancomycin 125mg oral 6-hourly for 14 days should be started.

If severity markers are absent, metronidazole 400mg oral 8-hourly is initiated. If there is improvement after 3-5 days, this treatment is continued for 10-14 days. When the improvement is not evident after 3-5 days or CDI is considered severe, vancomycin 125mg oral 6-hourly together with metronidazole 500mg intravenous 8-hourly are administered for 14 days.

Clearance testing is not advised in view of the potential for remainig toxin positive for weeks after symptoms have subsided. Repeat testing in confirmed positive cases should be carried out if symptoms have recurred after initial successful treatment. Repeat sampling in confirmed positive cases hould not be performed within 10 days of a positive result.

A form is to be filled in for *C. difficile* toxin positive cases as part of the European Surveillance of *Clostridium difficile* infections (Appendix 3).

1.17 Aims of the study

The aims of this study were to:

- Propose a framework for *C. difficile* culturing and antibiotic sensitivity testing in a clinical setting. This will allow selection of the appropriate antimicrobial to which the isolated strain shows sensitivity avoiding treatment failure, identification of resistant strains and investigation of local strains. This will shorten length of stay in hospital and involve further hospital cost reduction. Currently, there is no standardization for the culturing of *C. difficile* in the clinical setting.
- Assess current management of patients presenting with CDI at MDH and SAMOC: local compliance and patient outcomes.
- Provide information to enable an optimization of the current "Algorithm for *Clostridium difficile* infection investigation and results interpretation in adults" at MDH and SAMOC; upon identification of clinical issues or areas of improvement.
- Gather epidemiological data. This will provide an overview of the current local situation for CDI, allow comparison with different countries and reinforce local epidemiological studies.

CHAPTER 2

METHODOLOGY

Medical records of patients meeting the inclusion criteria were reviewed. Management was assessed and the collected information was statistically analysed to identify risk factors for *C. difficile* active infection or carriage. Available epidemiological data related to CDI from 2015 and 2016 was statistically analysed.

Approval from the University Research Ethics Committee (UREC) was obtained (Appendix 4). Publications of methods for culturing *C. difficile* and antibiotic sensitivity testing were reviewed and estimates for the materials considered necessary to run *C. difficile* culturing and antibiotic sensitivity testing in the clinical setting were requested from the local providers. A feasibiltity study for culturing *C. difficile* and antibiotic sensitivity testing was carried out.

2.1 *Clostridium difficile* culturing and antibiotic sensitivity testing

A total of 16 publications of methods for culturing *C. difficile* and antibiotic sensitivity testing were reviewed (George *et al,* 1979; Gresser *et al,* 1984; Delmee, 2001; Cowden *et al,* 2008; Eastwood *et al,* 2009; Schmidt and Gilligan, 2009; Perry *et al,* 2010; Noren *et al,* 2011; Peterson *et al,* 2011; Rennie *et al,* 2012; Tyrrell *et al,* 2013; Kim *et al,* 2014; Lister *et al,* 2014; Shin and Lee, 2014; Standards Unit, 2014; Galea *et al,* 2015) to help propose a framework for *C. difficile* culturing and antibiotic sensitivity testing in the clinical setting.

Cycloserine-cefoxitin-fructose-egg yolk agar (CCFA) was selected as the culturing media since it shows higher sensitivity and cost-effective performance for isolation of *C. difficile.* On this culturing media, colonies are yellow, with a ground-glass aspect and circular with a slightly filamentous edge. The colonies are even with a rounded rise, are lipase and lecithinase negative and show a distinctive golden-yellow fluorescence when

analysed under long-wave ultraviolet light (Holdeman *et al,* 1977; George *et al,* 1979; Jousimies-Somer *et al,* 2002).

Antimicrobial sensitivity testing allows selection of a suitable antimicrobial to which the isolated strain shows susteptibility. MIC Evaluator® strips by Thermo Fisher Scientific, Liofilchem® MIC test strips by Liofilchem s.r.l and Etest® by BioMerieux are available on the market. Etest® by BioMerieux[2] is the brand locally imported and it was selected for this proposed standard procedure. Etest® strips are available for more than 100 antimicrobials. The antibiotics suggested for the susceptibility test are cefotaxime, erythromycin, levofloxacin, meropenem, metronidazole, moxifloxacin, piperacillin-tazobactam, vancomycin and clindamycin. These antibiotics were selected based on review of previous studies to allow comparison in further investigations and in view of the limited data available in antibiotic sensitivity testing for *C. difficile* (Drudy *et al,* 2007; Cowden *et al,* 2008; Weber *et al,* 2013).

A standard procedure to culture *C. difficile* and test for antibiotic sensitivity (Appendix 8) was developed following literature review and discussion with specialists in the field of bacteriology. This standard procedure was elaborated under the assumption that some consumables are already available in the clinical setting for other tests. Not included in cost estimate were: specimen containers, media bottles, petri dishes, centrifuge/vortex mixer, sterile loops, swabs, sterile saline, methylated spirit, absolute alcohol, anaerobic jars, disposable pastette, and distilled water or demineralized water.

Local suppliers were requested to provide estimates for the materials deemed indispensable to perform *C. difficile* culturing and antibiotic sensitivity testing in the local clinical setting. Cost estimates were gathered from three local suppliers in 2016. The cheapest option was selected given the condition that the same quality of the product was

[2] BioMerieux. Etest: Antimicrobial susceptibility testing. REF 9302553C. 2012. [Online] Available from: http://www.ilexmedical.com/files/E-test-Package-Insert/AST_WW.pdf [Accessed 12th March 2016]

offered by more than one provider. The quotations included consumables to grant test performance on up to 30 samples. This number of samples was deemed appropriate for a feasibility study based on the number of reported positive samples at a national level in the past year (2015-56 positive samples for *C. difficile* toxin A&B).

Cost of the treatment as per "Algorithm for *Clostridium difficile* infection investigation and results interpretation in adults" in Malta was studied. Price of the different treatments was identified in the Central Procurement and Supplies Unit (CPSU) database. Cost of the hospital stay per day in Malta was also established.[3] Costs of the treatment for CDI as per current local guidelines and estimated cost of hospital stay per day in Malta were futher compared to the cost of performing the culturing and antibiotic sensitivity testing of *C. difficile* in the same setting.

2.2 Assessment of the current local management

This phase was carried out between April 2016 and December 2016 at MDH and SAMOC. MDH is the acute general and teaching hospital in Malta and is located in Msida, Malta. MDH has 928 inpatient beds, 86-day care beds, 4172 clinical and support staff and provides clinical service to different specialities. SAMOC is a specialised teaching hospital for Oncology and Haemato-Oncology and was inagurated on the 22nd December 2014, in Msida, Malta. This center has 113 inpatients beds and 12 outpatient clinics.

All faecal specimens to be tested for suspicion of CDI in Malta under the National Health Service coverage are processed in the virology laboratory at MDH.

To allow assessment of the current local management and to be able to provide information for further updating or optimization of the recently implemented "Algorithm for *Clostridium difficile* infection investigation and results interpretation in adults", a data

[3]Anastasi A, 2016, personal communication, 27th September 2016

collection sheet (Appendix 5) was set up to gather information from patients' medical records. Patients were recruited after an informed consent sheet (Appendix 6) was signed. Patients recruited had to meet the inclusion criteria established: inpatients at MDH or SAMOC after the implementation of the new "Algorithm for *Clostridium difficile* infection investigation and results interpretation in adults", aged 18 years-old or older and having faecal specimens positive for GDH.

Name and Identification Document number were recorded to allow follow-ups in case of further tests and or/recurrent cases. Medications were classified in several groups following the Anatomical Therapeutic Chemical (ATC) Classification System (Appendix 7) whilst antibiotics were classified according to their class and independently addressed to facilitate data analysis. Recent antibiotic exposure was assessed in the active and carrier population. Recent exposure was considered up to 3 months prior to the presentation of symptoms.

Collected data was analysed and compared to assess relevance as risk factors for CDI. Gender, age, length of stay in hospital, previous use of probiotics, use of PPIs, use of H_2-receptor antagonists, the presence of chronic kidney disease, intubation of the patient, nasogastric feeding and recent gastrointestinal surgery or exploration were taken into consideration.

This data was analysed with the SPSS® version 24 program. The Chi-square test was used to assess the association between two categorial variables. One of these variables indicates whether the patient is *C. difficile* carrier or active, while the other variable indicates either the demographic information (age and gender) or some infection risk factor-related information. This test was performed to establish the risk factors linked to the development of an active infection or to *C. difficile* carriage. The potential risk factors

included in the statistical analysis were: gender, age, antibiotic exposure, onset of the infection (community or health care facility acquired), use of probiotics, gastric acid suppression (PPI, H_2-receptor antagonists), gastrointestinal perturbations (intubation of the patient, nasogastric feeding, gastrointestinal surgery or exploration), immunosuppression, underlying chronic kidney disease.

A logistic regression model was applied to identify the effect of all risk factors when considered collectively. The major limitation of the Chi-square test is that it investigates the outcome (being *C. difficile* carrier or active) and a single risk factor (categorical predictor). The goal of many research studies is to estimate collectively the effect of all these risk factors upon the outcome. One risk factor could be rendered important in explaining variation in the outcome, but would be rendered unimportant in the presence of other risk factors. To tackle this problem, a logistic regression model was fit, where the outcome is the dependent variable and all the risk factors and demographic variables are the predictors.

Other assessed factors were: reason for admission, ward in hospital, past medical history, isolation of the patient, number of *C. difficile* tests performed, CDI patients and carrier patients' management and adherence to the "Algorithm for *Clostridium difficile* infection investigation and results interpretation in adults".

2.3 Epidemiological study

To provide an overview of the current local epidemiological situation for CDI and allow further comparison, the Pathology Department at MDH was contacted to obtain records of patients tested positive for *C. difficile* toxin in 2015 and 2016. Number of patients, gender, age and ID card number to support identification of recurrent cases/test duplications was requested. This provided information to assess the impact of the

"Algorithm for *Clostridium difficile* infection investigation and results interpretation in adults" implementation.

This data was statistically analysed by a difference of two proportions calculator. The number of affected individuals each year, first according to gender and then according to age, was compared to the total Maltese population. Data of the total Maltese population was obtained from the National Statistics Office in Malta (NSO). For the gender comparison, the total Maltese population was divided into two groups under the assumption that the gender in equally distributed in Malta. For the age comparison, the affected population was divided into two groups: ≤65 years-old and >65 years-old. The total population was grouped following this classification to allow comparison.

CHAPTER 3

RESULTS

3.1 *Clostridium difficile* culturing and antibiotic sensitivity testing

CCFA was selected after comparison with chromogenic medium, supplemented Brucella agar, and Fastidious Anaerobe Agar (Perry *et, al.* 2010; Peterson *et al,* 2011; Tyrrell *et al,* 2013; Weber *et al,* 2013; Kim *et al,* 2014).

American Type Culture Collection (ATCC) strains of *C. bifermentans, C. sordelii and C. difficile* were selected to be equally cultured for each sample as reference strains and assist in the identification of the *C. difficile* isolates. The use of ATCC strains aims to ensure quality control in the procedure (ATCC, 2016).

C. difficile isolates can be identified by colonial morphology on fastidious anaerobic agar culturing media and confirmed by the emission of green-yellow fluorescence under long-wave ultraviolet light. In the absence of a long-wave ultraviolet light locally and being CCFA the selected culturing media, identification by API 20A strips was suggested. This identification method shows a turnaround time of 24 hours, high reliability and accuracy when performed in pure culture (Gresser *et al,* 1984; Knoop *et al,* 1993).

The standard procedure (Appendix 8) was suggested in consideration of the absence of standards for the culturing and antibiotic sensitivity testing of *C. difficile* in the studied clinical setting.

The estimates were made for 30 stool samples (Table 3.1).

Table 3.1 Prices of consumables needed to run culturing and antibiotic sensitivity testing of *C. difficile* on a clinical setting for 30 samples

Product		**Price breakdown (€)**	**Total Price (€)**
CCEY (with egg yolk)	Clostridium Braziers Agar base (500g)	70.00	123.00
	Egg Yolk Emulsion (100ml per vial: 5 vials x 100ml)	53.00	
API 20 A	API 20 A (50 strips + 50 media)	320.00	790.64
API consumables	Mineral oil	5.29	67.43
	BCP	9.30	
	HER	10.24	
	XYL	10.71	
	McFarland Standard (12-week shelf life)	31.89	
Brucella broth 500g		28.50	28.50
Brucella agar plus haemin and vitamin K		26.50	26.50
Etest® strips	Cefotaxime (100 units)	324.74	2922.66
	Erythromycin(100 units)	324.74	
	Levofloxacin(100 units)	324.74	
	Meropenem(100 units)	324.74	
	Metronidazole(100 units)	324.74	
	Moxifloxacin(100 units)	324.74	
	Piperacillin-Tazobactam(100 units)	324.74	
	Vancomycin(100 units)	324.74	
	Clindamycin (100 units)	324.74	

The materials considered consumables were included in the estimates as expenses to run the *C. difficile* culturing and antibiotic sensitivity testing on a daily basis in a clinical setting. An initial investment for the ATCC strains, reference strains for quality control performance and API Web-Identification software is necessary, considering these components need to be purchased only once to allow a continuous test performance (Table 3.2). The cost for the reference strains used in the quality control of the antibiotic susceptibility testing, can vary depending on the agreement with the *C. difficile* reference laboratory.

Table 3.2 Initial costs to run culturing and antibiotic sensitivity testing of *C. difficile*

Product	**Price (€)**
API Web-Identification software	470.64
ATCC strains of *C.difficile, C.bifermentans* and *C.sorderlii*	791.00
Reference strains for quality control performance	*Upon agreement*

The final cost of culturing and antibiotic sensitivity testing for *C. difficile* in a local clinical setting for 30 samples is 3489.09 Euro which equates to 116.30 Euro per sample. This will grant a continuous test performance on a daily basis with up to 30 samples, provided that a first investment for the non-consumables listed in table 3.2 is made.

The local cost of treatment for CDI was identified for the year of 2016 (Table 3.3).

Table 3.3 Wholesale selling prices per unit and per treatment in euro as per 2016 in Malta

Drug	Cost per unit (€)	Cost per treatment (€)
Metronidazole 400mg oral tablets	0.25	7.5 (10 days) 10.5 (14 days)
Metronidazole 500mg solution for injection	0.94	28.29 (10 days) 39.48 (14 days)
Metronidazole 200mg/5ml suspension	18.40 (100ml)	According to weight 15 -30 mg/kg/day divided in 2-3 doses for 7 days
Vancomycin 125 mg oral capsules	5.13	287.50 (14 days)
Vancomycin 500mg powder for infusion for solution (to be administered orally)	6.30 (10ml vial)	352.80 (14 days)

Vancomycin 500mg powder for infusion for solution was included in view of the possibility of its oral administration as an alternative in the absence of vancomycin 125mg oral capsules.

The CPSU provided the cost of hospital stay in Malta, resulting in an average of €175 per day in 2016.[4]

3.2 Assessment of the current local management of CDI

Out of a total of 241 patients (GDH positive), 130 met the inclusion criteria; of which 67 patient medical records were reviewed. Sixty-three patient medical records were not reviewed when failing at obtaining patient informed consent due to decease of the patient, transfer of the patient to a different hospital or discharge of the patient. Out of the 67 patient medical records that were reviewed, 23 were reported as *C. difficile* active infection cases, 42 as carriers and 2 patients were excluded due to mixed results issued by the virology laboratory at MDH (Figure 3.1). Characteristics of the active and carrier population are shown in tables 3.4 and 3.5.

[4]Anastasi A, 2016, personal communication, 27th September 2016

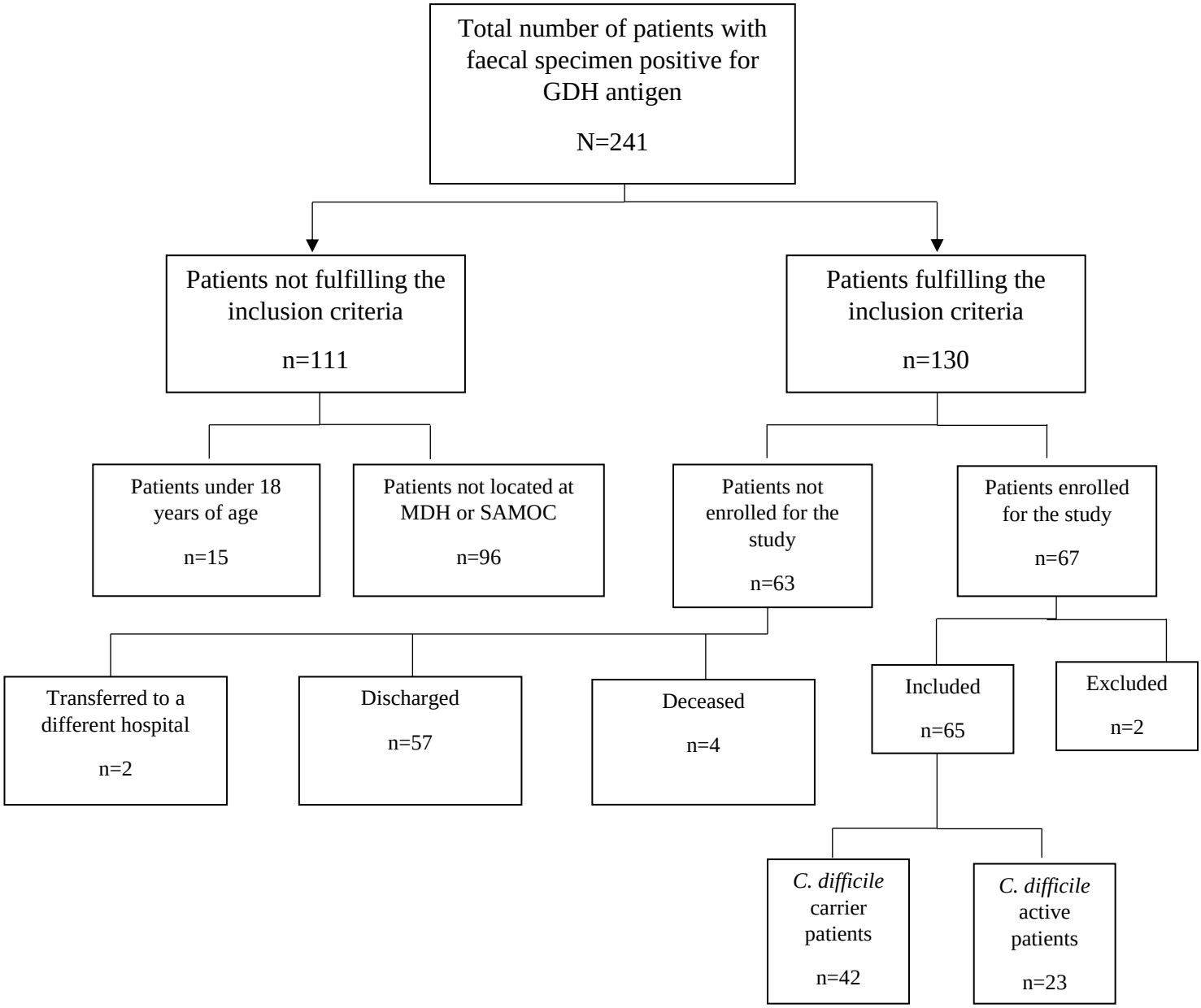


Figure 3.1 Patient data

Table 3.4 Characteristics of the *C. difficile* carrier population

Characteristic of *C. difficile* carrier patients		**Number/Percentage of the population (n=42)**
Age	18-25 years	3 (7%)
	26-35 years	3 (7%)
	36-45 years	3 (7%)
	46-55 years	5 (12%)
	56-65 years	4 (10%)
	66-75 years	11 (26%)
	76-85 years	8 (19%)
	86-95 years	5 (12%)
Race	White Caucasian	41 (98%)
	African American	0 (0%)
	Asian/Pacific Islander	1 (2%)
	Native American	0 (0%)
Gender	Male	18 (43%)
	Female	24 (57%)

Table 3.5 Characteristics of the *C. difficile* active population

Characteristic of *C. difficie* active patients		**Number/Percentage of the population (n=23)**
Age	18-25 years	1 (4%)
	26-35 years	4 (17%)
	36-45 years	0 (0%)
	46-55 years	3 (13%)
	56-65 years	1 (4%)
	66-75 years	7 (3%)
	76-85 years	5 (22%)
	86-95 years	2 (9%)
Race	White Caucasian	22 (96%)
	African American	1 (4%)
	Asian/Pacific Islander	0 (0%)
	Native American	0 (0%)
Gender	Male	9 (39%)
	Female	14 (61%)

The following graph (Figure 3.2) shows a comparison in age of *C. difficile* active and carrier patients in which the maximum number of patients belonging to the 66-75 years-old group in both groups.

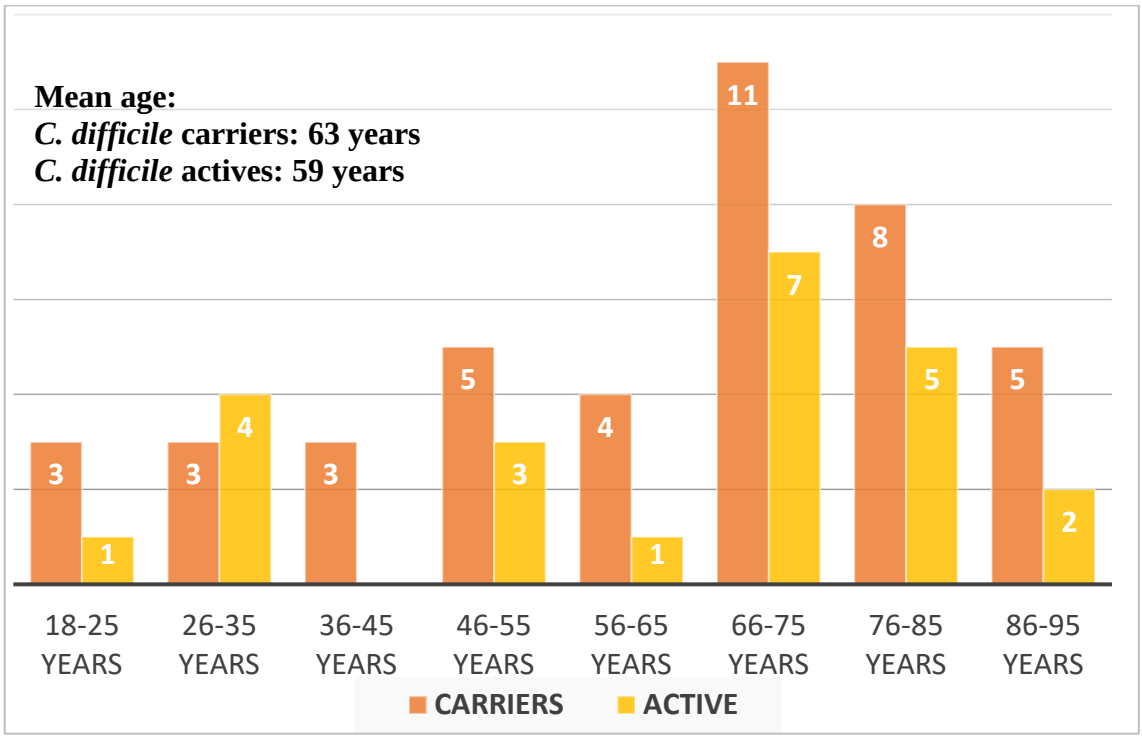


Figure 3.2 Age of studied population (N=65)

Table 3.6 shows the antibiotic exposure in the studied population, where antibiotics are grouped by class.

Table 3.6 Antibiotic exposure in the studied population

Population	**Number of antibiotics**		**Class**	**Number of cases**
C. difficile active	Zero	1 (4%)	Penicilin/Penicillin combinations	12
	One	9 (39%)		
	Two	6 (26%)	Fluoroquinolones	8
	Three	2 (9%)	Aminoglycosides	5
	≥Four	5 (22%)	Carbapenems	4
			Glycopeptides	2
			Lincosamide (Clindamycin)	4
			Cephalosporin 3nd generation	3
			Sulfonamides	2
			Cephalosporin 2nd generation	1
			Metronidazole	1
			Macrolide	1
			Nitrofuran	1
C. difficile carriers	Zero	11 (26%)	Penicillin/Penicillin combinations	24
	One	13 (31%)	Carbapenems	9
	Two	6 (14%)	Fluoroquinolones	7
	Three	7 (17%)	Aminoglycosides	6
	≥ Four	5 (12%)	Sulfonamides	6
			Cephalosporines 3rd generation	4
			Lincosamides (Clindamycin)	3
			Metronidazole	2
			Nitrofuran	2
			Tetracyclines	2
			Cephalosporin 2nd generation	1
			Fosfomycin	1
			Tigecycline	1

Figure 3.3 shows the percentage of patients exposed to antibiotic treatment in the past three months before the development of the symptoms related to CDI.

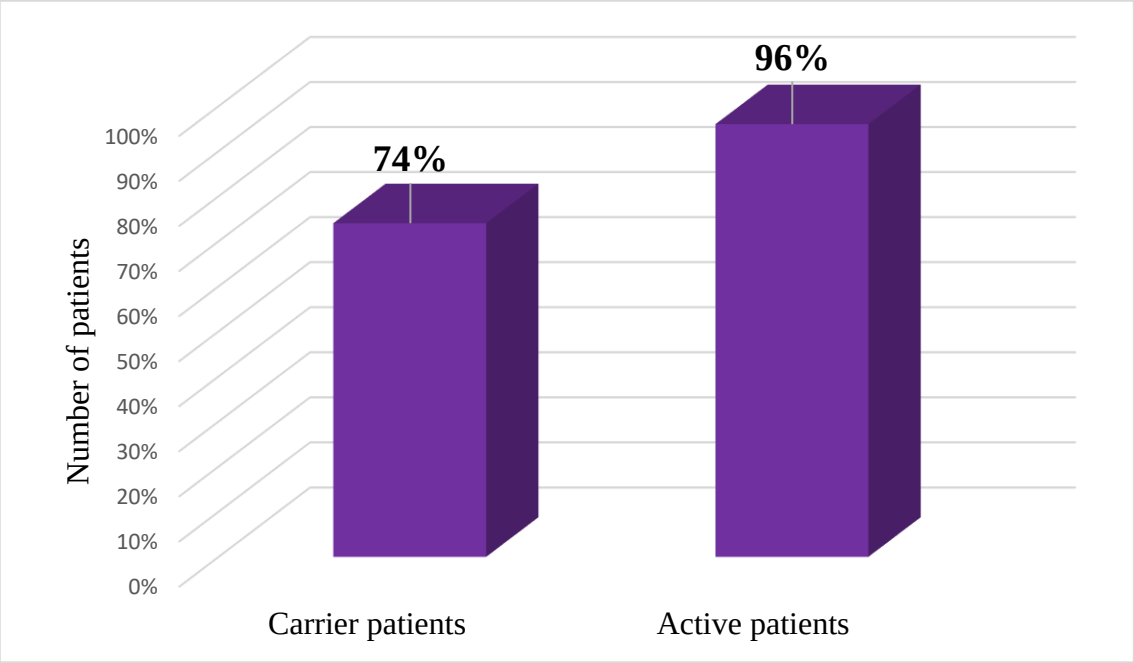


Figure 3.3 Antibiotic exposure in *C. difficile* carrier patients (n=42) and active patients (n=23)

A classification of the cases was carried out according to the onset of the infection (Table 3.7). The majority of the cases were reported to have a health care facility onset.

Table 3.7 Classification of *C. difficile* infection according to onset of symptoms

	Onset classification	**Number**	**Mean age (years)**
C. difficile carrier patients (n=42)	Community	14 (33%)	67
	Indeterminate	0 (0%)	N/A
	Health care facility	28 (67%)	51
C. difficile active patients (n=23)	Community	7 (30%)	59
	Indeterminate	1 (4%)	87
	Health care facility	15 (65%)	62

C. difficile active and carrier cases with a health care facility onset were further classified according to their length of stay in hospital upon presentation of CDI symptoms to assess its impact as a risk factor (Table 3.7). Both groups showed an early onset of the infection for the majority of the population (3-20 days).

Table 3.8 Length of stay in hospital for carrier and active cases with a health care facility onset of the infection

Length of stay in hospital (days)	***C. difficile* carrier patients n=28**	***C. difficile* active patients n=14**
3-20	22	8
21-40	2	1
41-60	1	3
61-80	1	0
81-100	0	1
101-120	0	0
121-140	0	0
141-160	0	1
161-180	1	0
>181	1	0

The use of probiotics while symptoms were present among both populations was evaluated to identify the role and potential beneficial input in CDI and carriage (Figures 3.4 and 3.5). The reported use of probiotics was in symptomatic patients.

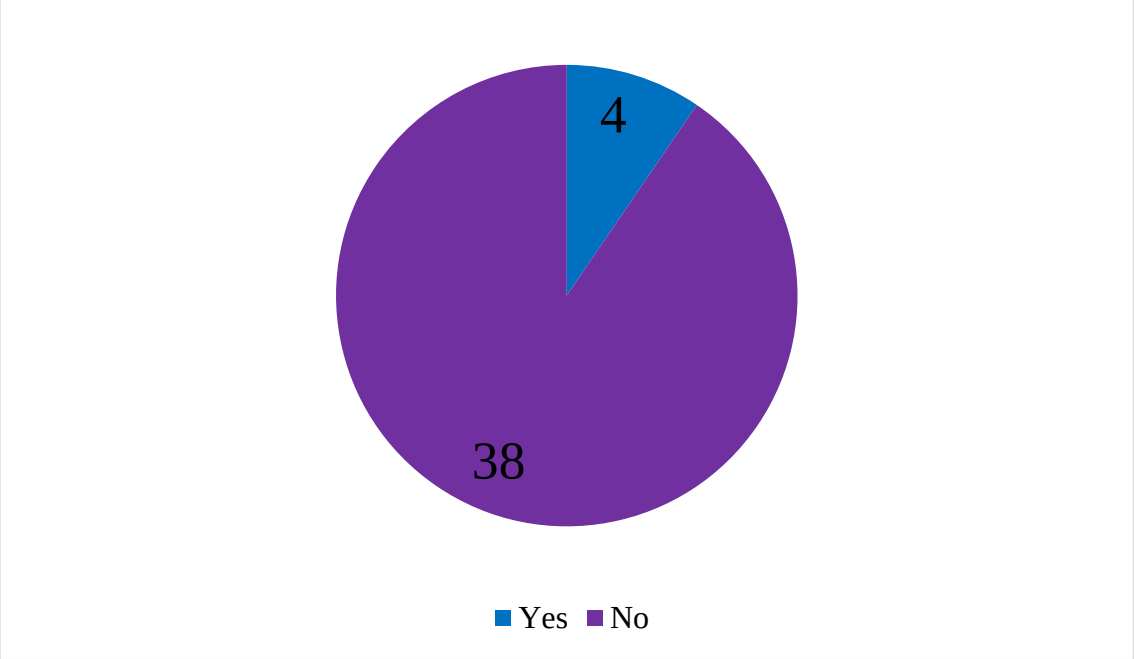


Figure 3.4 Use of probiotics among *C. difficile* carrier patients (n=42)

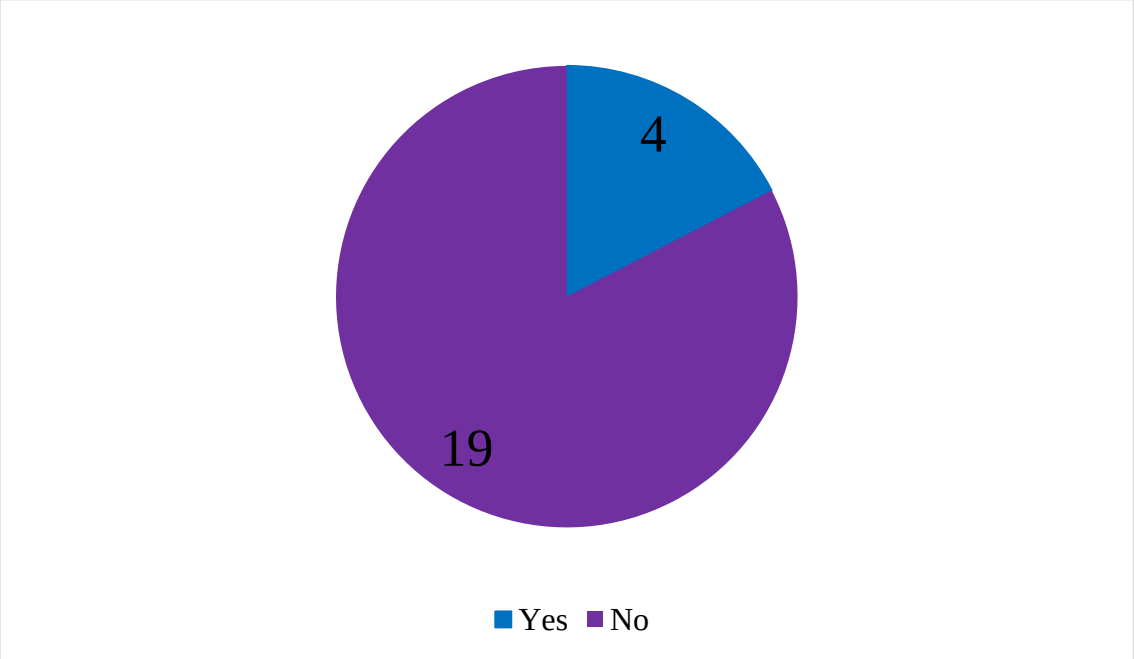


Figure 3.5 Use of probiotics among *C. difficile* active patients (n=23)

Gastric acid supression has been reported as a risk factor for CDI. Its role on *C. difficile* carriage has not been described. Patients' gastric acid suppression therapy is represented in figures 3.6 and 3.7.

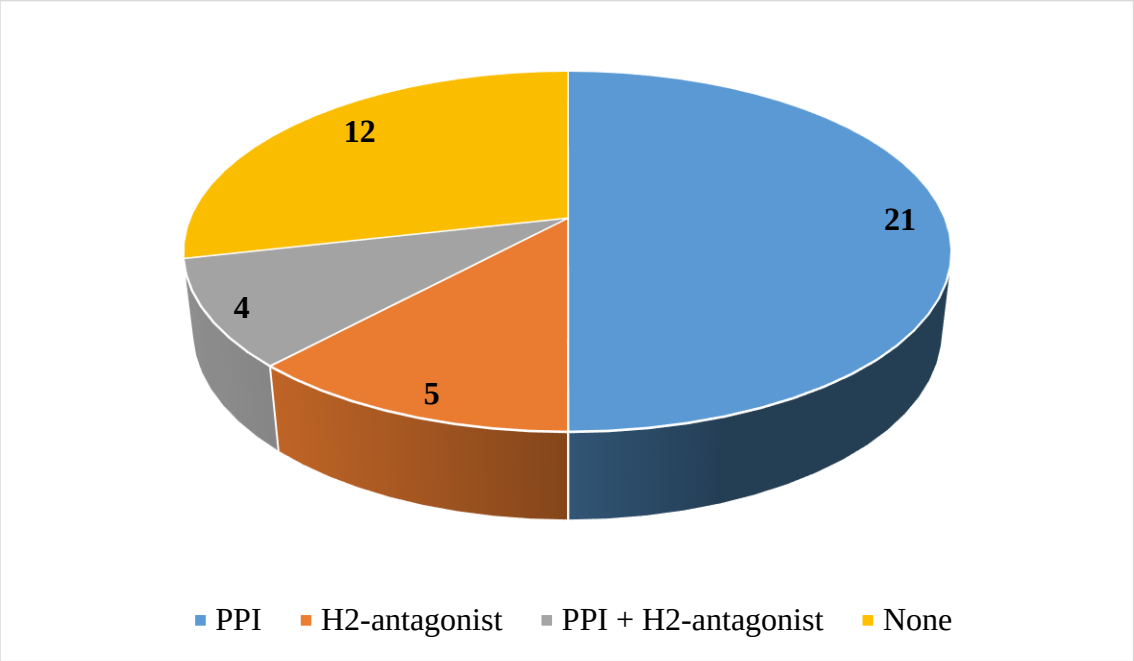


Figure 3.6 Gastric acid suppression in *C. difficile* carrier patients (n=42)

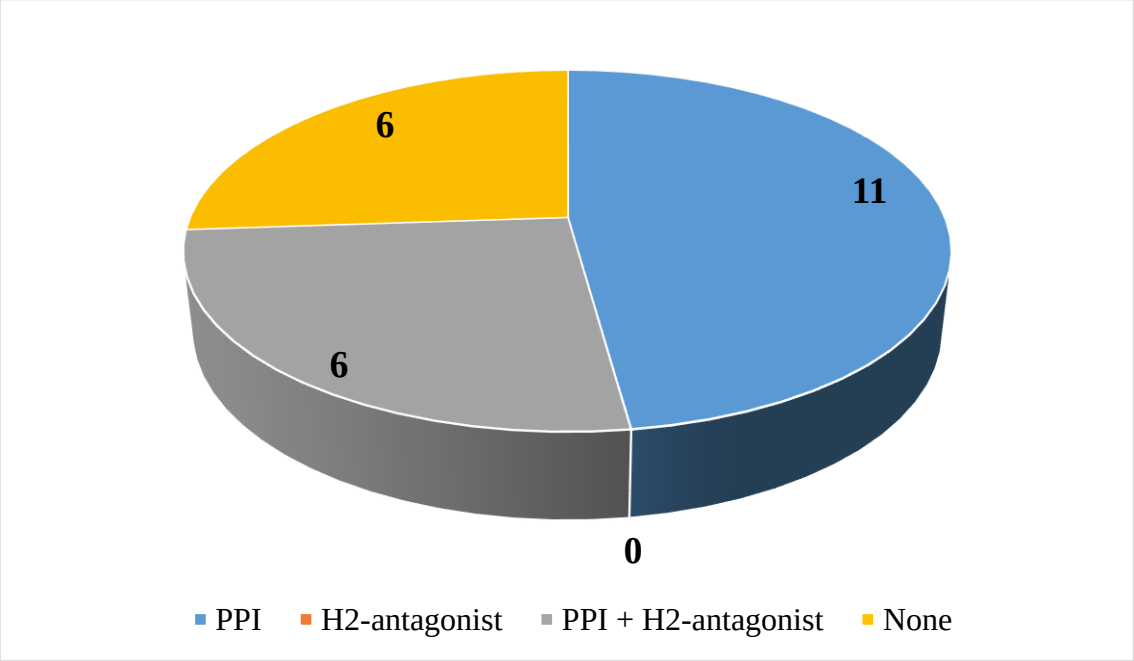


Figure 3.7 Gastric acid suppression in *C. difficile* active patients (n=23)

Perturbation of the gastrointestinal tract was assessed. Three categories were included: patient intubation, nasogastric tube (NGT) feeding and gastrointestinal surgery/internal examination in the past 3 months. The registered gastrointestinal interventions and or examinations were: abdomen hernia, hemicolectomy, ileostomy, cholecystectomy, esophageal varices, gastrectomy, flexible sigmoidoscopy, colectomy, appendectomy and stoma. These factors are represented in figures 3.8 and 3.9, showing that 43% of the *C. difficile* carrier patients and 57% of the *C. difficile* active patients suffered perturbations affecting their gastrointestinal tract.

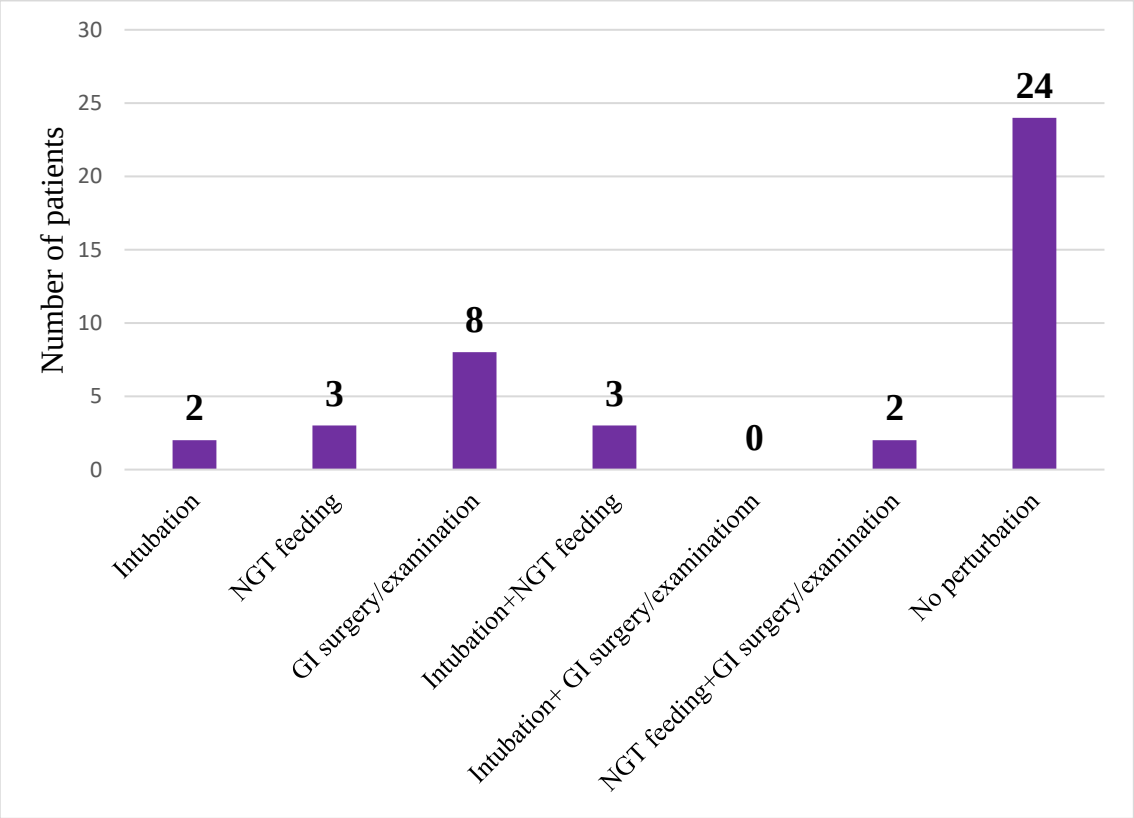


Figure 3.8 Gastrointestinal perturbations in *C. difficile* carrier patients (n=42)

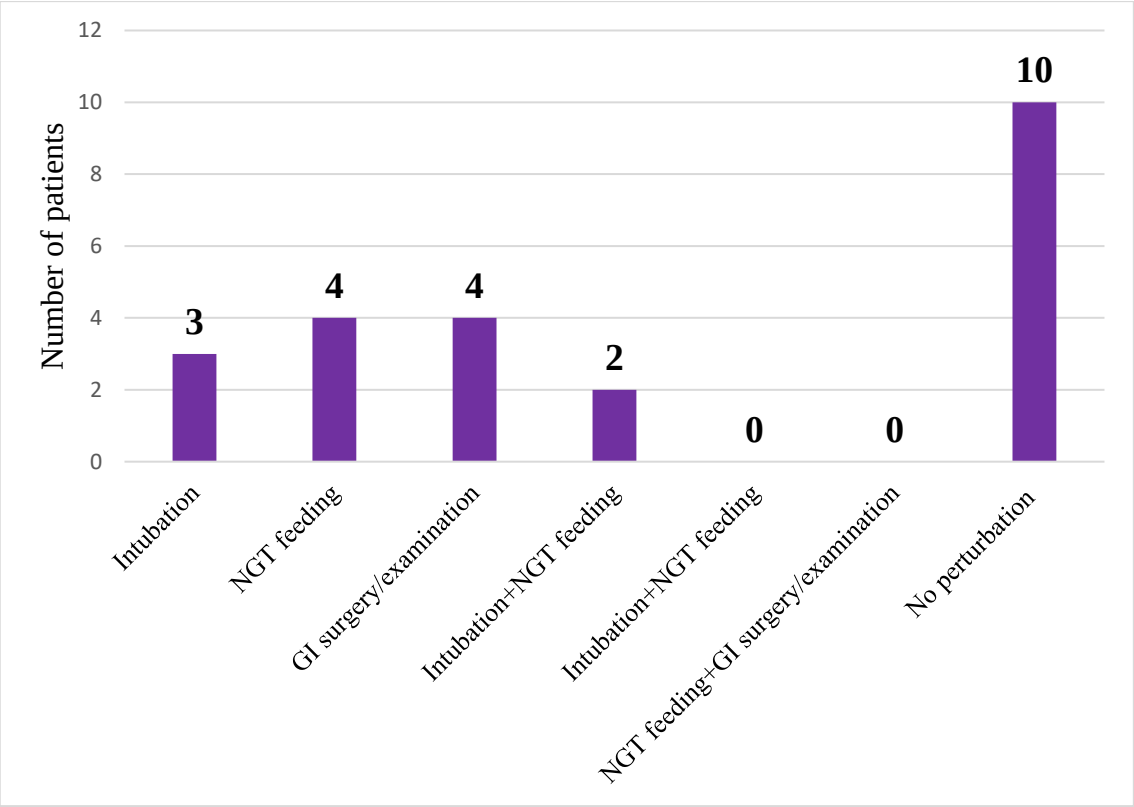


Figure 3.9 Gastrointestinal perturbations in *C. difficile* active patients (n=23)

The presence of underlying chronic kidney pathology was assessed as a risk factor. Three patients in the carrier group and 5 in the active group were reported, which equals to 8% in the carrier population and 22% in the active population.

The reason for admission in hospital of the studied population are listed on the following table (table 3.9).

Table 3.9 Reason for admission in hospital

***C. difficile* carrier patients n=42**		***C. difficile* active patients n=23**	
Reason	**No. of patients**	**Reason**	**No. of patients**
Allergic reaction	2	Constipation	1
Asthenia	1	Deteriorariation following chemotherapy	1
Bleeding haemorroids	1	Diarrhoea	2
Chemotherapy	3	Edema	1
Diarrhoea/loose stools	4	Epilepsy	1
Edema	1	Fever	2
Epigastric pain	2	Hypertension	1
Epilepsy	1	Infection complication	4
Fever	2	Lethargy	1
Hypercalcemia	1	Painful lymph node	1
Infection complication	4	Red blood cells transfusion	1
Pain	1	Renal transplant complication	1
Polytrauma	5	Sepsis	2
Sepsis	1	SOB	1
Shortness of breath (SOB)	2	Suicidal attempt	1
Surgery	2	Urinary tract infection (UTI)	1
Transient ischemic attack (TIA)	1	Vomiting + loose stools 1	1
Vomiting	4	Not reported	1
Vomiting + loose stools	2		
Not reported	2		

Immunosuppresion is another relevant risk factor for CDI. Patients were considered immunosuppressed when on immunosuppressant treatment or autoimmune disease.

The number of patients affected by immunosuppression are represented in figures 3.10 and 3.11.

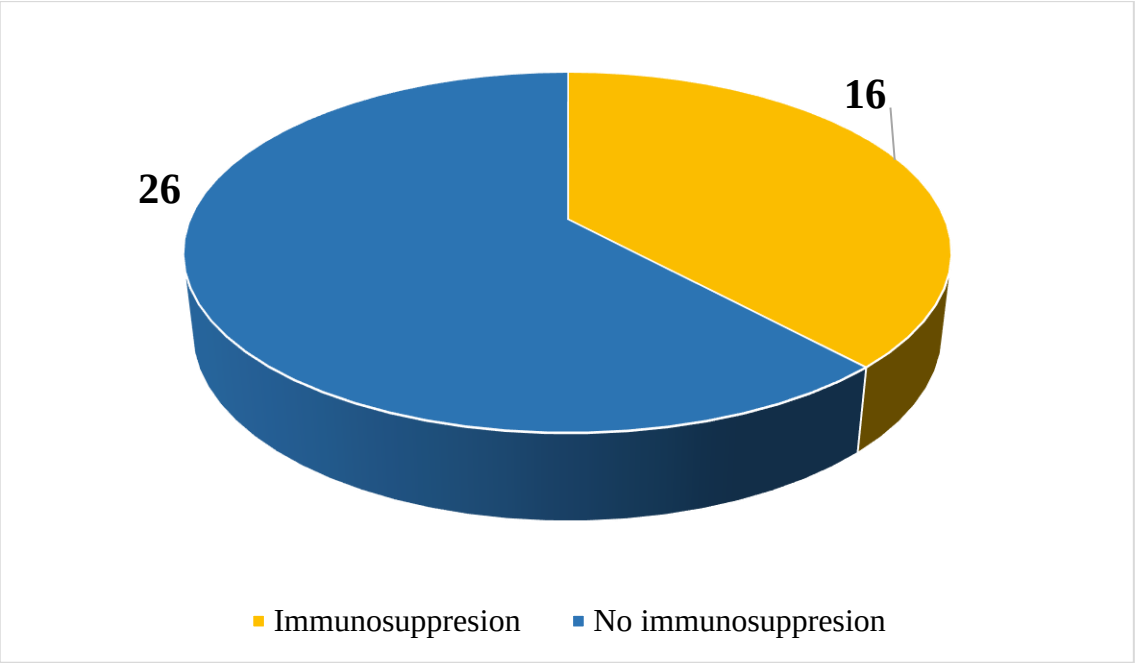


Figure 3.10 Immunosuppresion in *C. difficile* carrier patients (n=42)

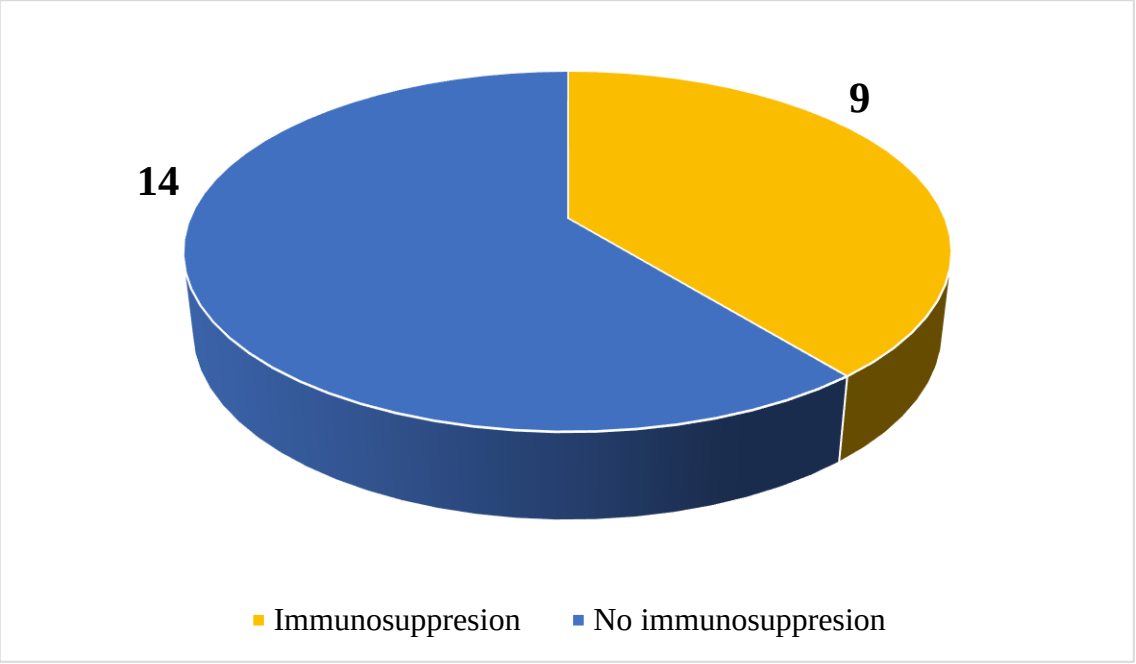


Figure 3.11 Immunosuppresion in *C. difficile* active patients (n=23)

Chronic medication was reviewed and classified for *C. difficile* carriers and active patients to aid the identification of any other potential modifiable risk factor to acquire *C. diffcile* (Table 3.10).

Table 3.10 Chronic medication of the studied population

Drug ATC classification	*C. difficile* carriers n=42	*C. difficile* active n=23
ACE inhibitor	13 (31%)	8 (35%)
Adrenergic inhalant	4 (10%)	2 (9%)
Alpha-adrenoreceptor antagonist	0 (0%)	2 (9%)
Angiotensin II antagonist	3 (7%)	1 (4%)
Antiarrhythmic	2 (5%)	1 (4%)
Anti-dementia drug	1 (2%)	1 (4%)
Antidepressant	8 (19%)	3 (13%)
Antiemetic and antinauseant	5 (12%)	1 (4%)
Antiepileptic	4 (10%)	4 (17%)
Antifungals for systemic use	9 (21%)	3 (13%)
Antiglaucoma preparation	3 (7%)	1 (4%)
Antigout preparation	2 (5%)	3 (13%)
Antihistamine for systemic use	0 (0%)	2 (9%)
Antimetabolite	2 (5%)	2 (9%)
Antineoplastic agent	5 (12%)	1(4%)
Antiparkinson drug	0 (0%)	1 (4%)
Antipsychotic	2 (5%)	1 (4%)
Antithrombotic agent	7 (17%)	9 (39%)
Beta blocking agents	8 (19%)	2 (9%)
Biphosphonate (oral)	0 (0%)	1 (4%)
Blood glucose lowering drugs	11 (26%)	4 (17%)
Calcium	7 (5%)	3 (13%)
Capillary stabilizing agents	1 (2%)	0 (0%)
Cardiac glycosides	2 (5%)	1 (4%)
Contact laxative	0 (0%)	1 (4%)
Corticosteroid for systemic use	2 (5%)	3 (13%)
Cytotoxic antibiotics and related	0 (0%)	2 (9%)
Direct acting antiviral	7 (17%)	3 (13%)
Drug for functional gastrointestinal disorders, propulsive	0 (0%)	1 (5%)
Drug for obstructive airway diseases, inhalant	1 (2%)	2 (9%)
Drugs for peptic ulcer and gastro-oesophageal reflux disease (GORD)	9 (21%)	6 (26%)
Drugs used in benign prostatic hypertrophy	2 (5%)	0 (0%)
Folic acid and derivatives	3 (7%)	4 (17%)
High-ceiling diuretics	12 (29%)	5 (19%)
Hormone antagonist and related agents	0 (0%)	1 (4%)
Hypnotics and sedatives	3 (7%)	5 (22%)
Intestinal antiinflamatory agents	1 (2%)	0 (0%)

Table 3.10 Chronic medication of the studied population

Drug ATC classification	*C. difficile* carriers n=42	*C. difficile* active n=23
Immunostimulant	2 (5%)	1 (4%)
Immunosuppressant	2 (5%)	4 (17%)
Insulin and analogue for injection	10 (24%)	3 (13%)
Lipid modifying agents	8 (19%)	10 (43%)
Low-ceiling diuretics	1 (2%)	3 (13%)
Muscle relaxant	0 (0%)	1 (4%)
Opioid	2 (5%)	1 (4%)
Osmotically active laxatives	4 (10%)	3 (13%)
Other analgesic and antipyretic	9 (21%)	2 (9%)
Other antineoplasic agents	1 (2%)	1 (4%)
Other cardiac preparations	3 (7%)	0 (0%)
Oral iron preparations	4 (10%)	3 (13%)
Other nervous system drug	0 (0%)	2 (9%)
Potassium	2 (5%)	0 (0%)
Potassium-sparing agents, diuretics	1 (2%)	0 (0%)
PPI	25 (60%)	17 (74%)
Psicoanaleptic antidepressant	0 (0%)	2 (9%)
Selective calcium channel blocker	8 (19%)	3 (13%)
Synthetic anticholinergic	1 (2%)	2 (9%)
Thyroid preparations	3 (7%)	1 (4%)
Vasodilator used in cardiac diseases	5 (12%)	2 (9%)
Vitamin B12	1 (2%)	0 (0%)
Vitamin B-complex combinations	0 (0%)	1 (4%)
Vitamin D and analogues	0 (0%)	1 (4%)

Adherence to local algorithm was evaluated by recording: testing adequacy, treatment according to algorithm, isolation of the patient, stool charting and discontinuation of non-clostridial antibiotics, antimotility agents and gastric acid suppression when possible.

In all cases symptoms were present upon stool specimen submission (*C. difficile* active and carrier patients). Clearance testing is not recommended in view of the likelihood to remain toxin positive for weeks after symptoms have resolved. In the studied population, clearance testing was performed in two cases. In these two instances, a first GDH positive/A & B toxin positive result was issued and a subsequent GDH positive/A &B toxin negative was reported.

More than one stool specimen was sent to the laboratory in the the same day in two cases. Submission of several samples in the same day is not recommended on the local algorithm.

The treatment administered was documented in all the studied cases. Treatment administered was in disagreement with the algorithm in nine occasions as presented in Table 3.11. Recommendation by the microbiologist for this treatment regimens was not reported.

Table 3.11 Recorded treatment regimens administered in discrepancy when compared with the local algorithm

Number of cases (n=9)	**Treatment administered**	**Treatment according to algorithm**
2 cases	Metronidazole 400mg ORAL 8-hourly + vancomycin 125mg ORAL 6-hourly	Metronidazole 500mg IV 8-hourly + vancomycin 125mg ORAL
2 cases	Vancomycin 2g IV 12-hourly (oral route available)	Metronidazole 500mg IV 8-hourly + vancomycin 125mg ORAL 6-hourly
1 case	Omeprazole 20mg ORAL 12-hourly + Domperidone 10mg ORAL 8-hourly	Metronidazole 400mg ORAL 8-hourly OR vancomycin 125mg ORAL 6-hourly
2 cases	Metronidazole 500mg IV 8-hourly (oral route available)	Metronidazole 400mg ORAL 8-hourly
2 cases	Ciprofoxacin 500mg ORAL 12-hourly	Metronidazole 400mg ORAL 8-hourly

Gastric acid suppression was continued in two cases. There was no disclosed indication for gastric acid suppression therapy in any of these two instances.

A total of 13 cases of non-adherence to the local algorithm were identified, corresponding to 2 faecal specimen submissions not according to the local algorithm, 9 treatment therapies not according to the algorithm and 2 gastric acid suppression therapy continuations not according to the local algorithm, resulting in a 21% non-compliance rate to this algorithm.

The presence of symptoms was assessed at the time when the laboratory issued the results. In nine cases, the presence of *C. difficile* was not reported on the doctor's notes following laboratory result notification and no actions were taken. These nine cases were *C. difficile* carriers. Results are documented in figures 3.12 and 3.13.

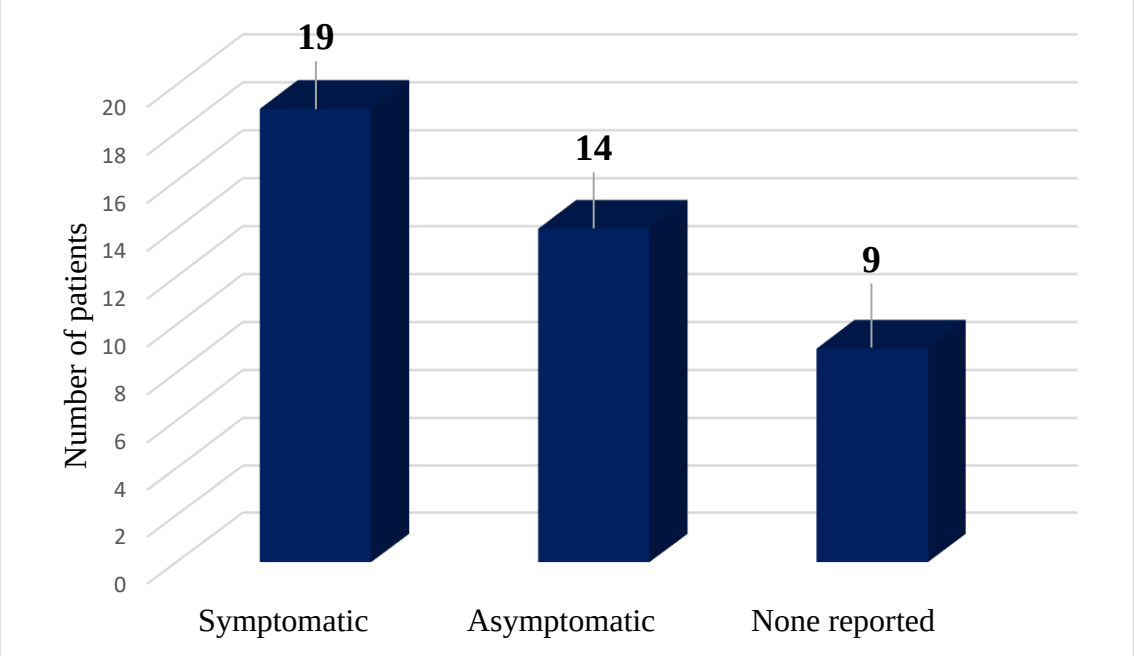


Figure 3.12 Manifestation of symptoms in *C. difficile* carrier patients (n=42)

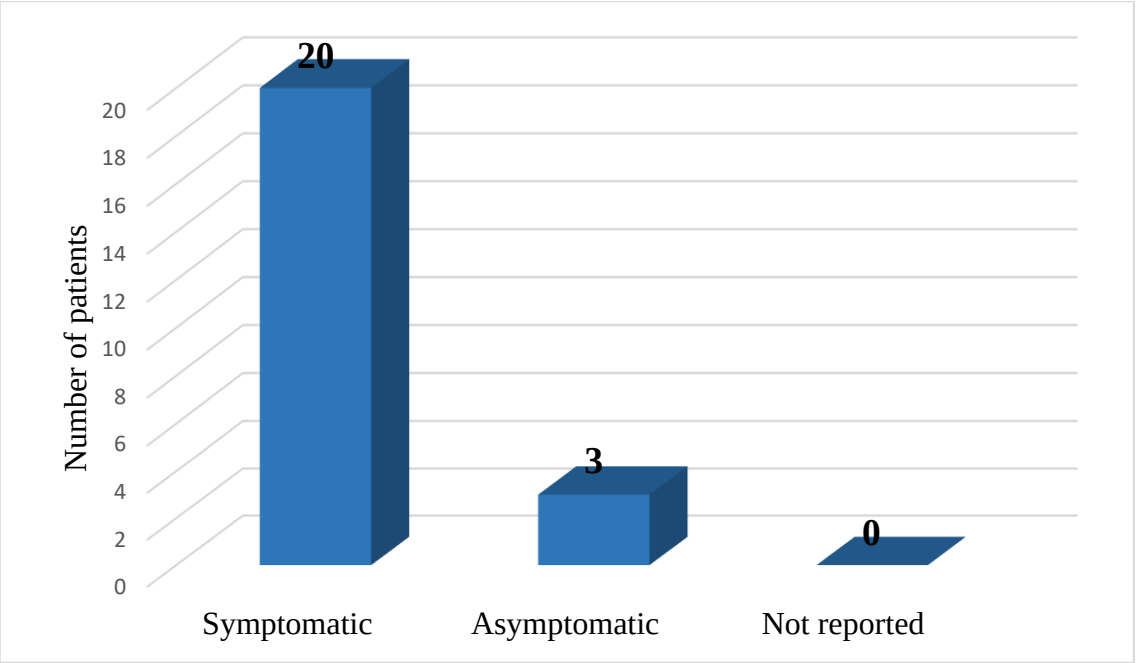


Figure 3.13 Manifestation of symptoms in *C. difficile* active patients (n=23)

Isolation of symptomatic patients is recommended and until free from diarrhoea for 48 hours. The isolation of patients is described in figures 3.14 and 3.15.

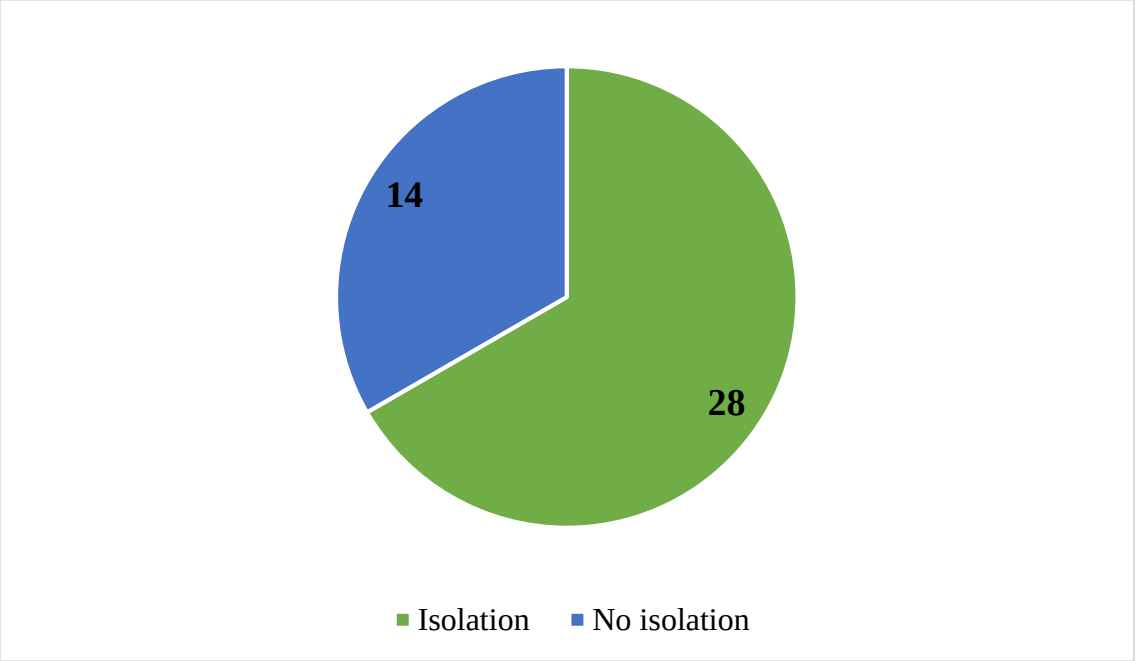


Figure 3.14 Isolation of *C. difficile* carrier patients (n=42)

Among the non-isolated fourteen *C. difficile* carrier patients, two were reported as symptomatic patients, five were not reported as *C. difficile* and seven were asymptomatic.

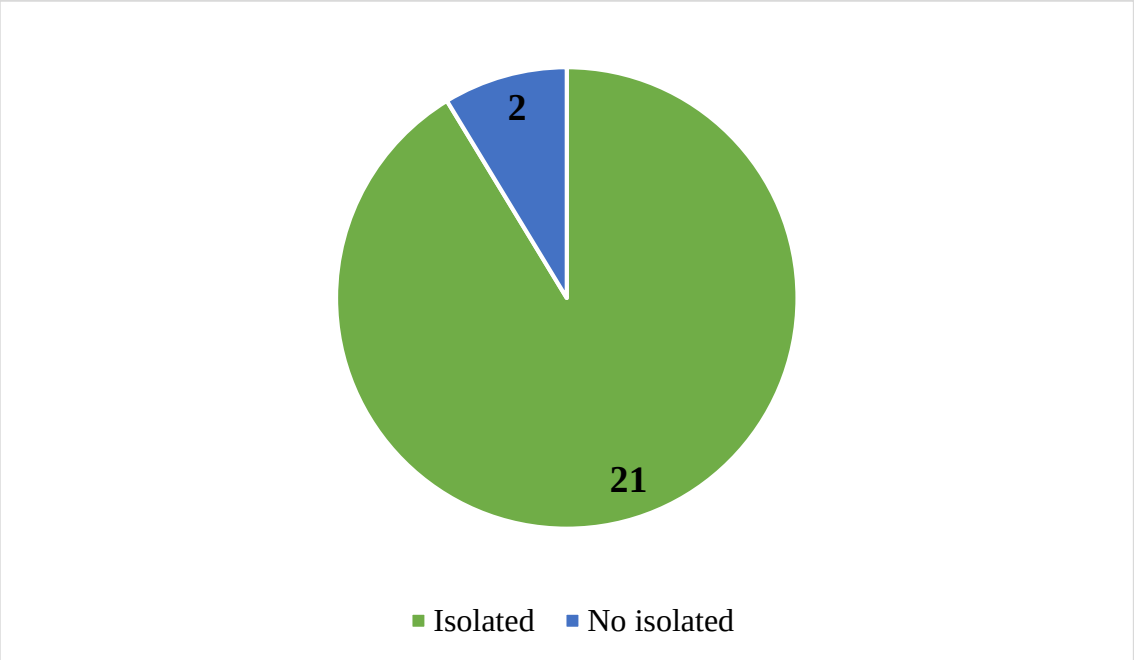


Figure 3.15 Isolation of *C. difficile* active patients (n=23)

There were two *C. difficile* active patients who were not isolated. These patients were asymptomatic.

Disinfection of the room by hydrogen peroxide fogging was performed following discharge, transfer of decease of all symptomatic patients according to the form attached to patients' files (Figure 1.1). Diarrhoea monitoring was carried out by Bristol stool chart (Appendix 1) on the symptomatic patients.

The chi-square test was carried out to identify the modifiable and non-modifiable risk factors to acquire the non-toxigenic *C. difficile* carriage or to progress to the active infection. This helped characterise the factors that will provide an individual with a higher risk to be a *C. difficile* carrier rather than an active patient and conversely.

The factors identified in this study as significant factors increasing risk to develop the active infection rather than carriage of *C. difficile* are antibiotic exposure (p=0.030) and chronic kidney disease (p=0.087). Chronic kidney disease was considered of relevance considering that the p–value is close to the level of significance and a logistic regression model was established to assess chronic kidney disease as a relevant factor to progress to *C. difficile* active infection. Tables 3.12 and 3.13 display these results.

Table 3.12 represents the results of the Chi-square test for antibiotic exposure as a risk factor. There is a greater percentage of *C. difficile* actives (95.7%) compared to the carriers (73.8%) who were exposed to antibiotics in the past three months. The p-value (0.030) is under the level of significance of 0.05 so the null hypothesis is rejected and the alternative hypothesis which states that a difference of 21.9% is significant with evidence that antibiotic exposure is a significant risk factor to be a *C. difficile* carrier or active is accepted. Exposure to antibiotics involves a higher risk to develop the active infection rather than carriage of *C. difficile.*

Table 3.12 Chi-square test result for antibiotic exposure as a risk factor

			Outcome		
			Active	Carrier	Total
Antibiotic exposure	Exposed	Count	22	31	53
		Percentage	95.7%	73.8%	81.5%
	Not exposed	Count	1	11	12
		Percentage	4.3%	26.2%	18.5%
Total		Count	23	42	65
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 4.710$, $p = 0.030$					

Table 3.13 displays the results of the Chi-square test for chronic kidney disease as a risk factor for CDI. There is a higher percentage (21.7%) of *C. difficile* actives compared to the carriers (7.1%) who suffered from chronic kidney disease. The p-value (0.0.087) is under the level of significance (0.05) and the null hypothesis is accepted, which implies that a difference of 14.6% is not significant with no evidence that chronic kidney disease is a significant risk factor for being a *C. difficile* carrier or active. This factor is still considered of relevance since the p-value is close to the level of significance.

Table 3.13 Chi-square test result for chronic kidney disease as a risk factor

			Outcome		
			Active	Carrier	Total
Chronic kidney disease	Yes	Count	5	3	8
		Percentage	21.7%	7.1%	12.3%
	No	Count	18	39	57
		Percentage	78.3%	92.9%	87.7%
Total		Count	23	42	65
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 2.934$, $p = 0.087$					

The results of the Chi-square test performed to assess the association between other studied risk factors and being a *C. difficile* carrier or active patient did not show statistical significance (Appendix 9). This risk factors are: gender, age, onset of the infection, use of probiotics, gastric acid suppression, gastrointestinal perturbations and immunosuppression. They were statistically analysed to assess their implication in CDI to progress to the active infection rather than carriage of *C. difficile.* They were not significant factors (Table 3.14)

Table 3.14 Risk factors with no statistical significance according to Chi-square test results

Risk factor	**p-value**
Gender	0.413
Age	0.771
Onset of the infection	0.619
Use of probiotics	0.662
Gastric acid suppression	0.831
Gastrointestinal perturbations	0.292
Immunosuppresion	0.935

The logistic regression model was established and is represented in Tables 3.15 and 3.16. The likelihood ratio tests showed that when all the risk factors are considered collectively, antibiotic exposure and chronic kidney disease are the factors that rendered more important in explaining the variations of the outcoume (being a *C. difficile* carrier or active). A patient who had a recent antibiotic exposure is at a higher risk of being a *C. difficile* active rather than a carrier. A patient who suffers from chronic kidney disease is at a greater risk of developing the active infection rather than being a carrier.

The logistic regression model identified antibiotic exposure as the best factor (predictor) of the outcome. Since it has the lowest p-value (0.024), this is followed by chronic kidney disease (p-value: 0.096). It should be noted that only antibiotic exposure is a significant risk factor because the p-value is less than the 0.05 level of significance.

The Odds ratio for recent antibiotic exposure (7.43) indicates that for a patient who was exposed to antibiotics, the odds that this patient is *C. difficile* active rather than carrier is 7.43 times that of a patient who was not exposed to antibiotics.

The odds ratio for chronic kidney disease (3.81) indicates that for a patient with chronic kidney disease, the odds that this patient is *C. difficile* active rather than carrier is 3.81 times that of a patient who does not suffer from chronic kidney disease.

Table 3.15 Likelihood ratio tests

		Likelihood Ratio Tests		
Effect	Deviance	Chi-Square	df	P-value
Intercept	56.005	0.000	0	.
Antibiotic exposure	61.080	5.075	1	0.024
Chronic kidney disease	58.779	2.774	1	0.096

Table 3.16 Parameter estimates

	B	Std. Error	Wald	df	P-value	Odds Ratio
Intercept	-0.573	.309	3.449	1	0.063	
Antibiotic exposure=Yes	2.006	1.097	3.340	1	0.068	7.430
Antibiotic exposure=No	0	.	.	0	.	.
Chronic kidney disease=Yes	1.337	.826	2.618	1	.106	3.808
Chronic kidney disease=No	0	.	.	0	.	.

To further analyse the potential combinations of these two factors and their risk to develop into an active infection rather than carriage, a mathematical formula with the components of the logistic regression model was developed (Table 3.17). P represents a *C. difficile* active patient and 1-p represents a *C. difficile* carrier. All the feasible combinations were calculated and results are displayed on table 3.18.

Table 3.17 Formula from the logistic regression model

$\log_e\left(\frac{p}{1-p}\right) = -0.573 + 2.006AE + 1.337KD$	
AE=1	Patient exposed to antibiotics
AE=0	Patient not exposed to antibiotics
KD=1	Patient suffers from chronic kidney disease
KD=0	Patient does not suffer from chronic kidney disease

Table 3.18 Results from applying formula to the four possible scenarios

	Result "p"	**Result "1-p"**
AE=1; KD=1	0.941	0.059
AE=1; KD=0	0.807	0.193
AE=0; KD=1	0.683	0.317
AE=0; KD=0	0.361	0.639

When AE=1 and KD=1, it is assumed that a patient was exposed to antibiotics and suffers from chronic kidney disease, in this case “p” is 0.941 and “1-p” is 0.059. The chance that this patient is *C. difficile* active is 94.1% and the chance that the patient is a *C. difficile* carrier is 5.9%.

When AE=1 and KD=0, it is assumed that a patient was exposed to antibiotics but does not suffer from chronic kidney disease, in this instance “p” is 0.807 and “1-p” is 0.193. The probability that this patient is *C. difficile* active is 80.7% and the probability that this patient is *C. difficile* carrier is 19.3%.

If AE=0 and KD=1, it is assumed that a patient was not exposed to antibiotics but he suffers from chronic kidney disease, in this case “p “is 0.683 and “1-p” is 0.317. The probability that this patient is *C. difficile* active is 68.3% and the probability that this patient is *C. difficile* carrier is 31.7%.

When AE=0 and KD=0, it is assumed that a patient was not exposed to antibiotics and does not suffer from chronic kidney disease, in this instance “p” is 0.361 and “1-p” is 0.639. The chance that this patient is *C. difficile* active is 36.1% and the probability that this patient is *C. difficile* carrier is 63.9%.

3.3 Epidemiological study

The totality of the Maltese population with A&B toxin positive result was studied to identify the prevalence of CDI in 2015 and 2016. Toxin positive cases from both years were compiled and analysed according to gender and age. Results are represented in table 3.19. All cases of recurrent symptoms following resolution after *C. difficile* treatment were incorporated among the recurrent cases in 2016, including *C. difficile* symptomatic carriers. *C. difficile* positive cases (2015 and 2016) according to gender is represented on figure 3.16.

Table 3.19 Epidemiological data in Malta in 2015 and 2016

	2015	**2016**
Specimens tested	1968	3391
A&B toxin positive samples	56	111
***C. difficile* active patients**	48	99
GDH+/A&B toxin negative samples	N/A	181
***C. difficile* carrier patients**	N/A	142
Recurrent cases	3	10

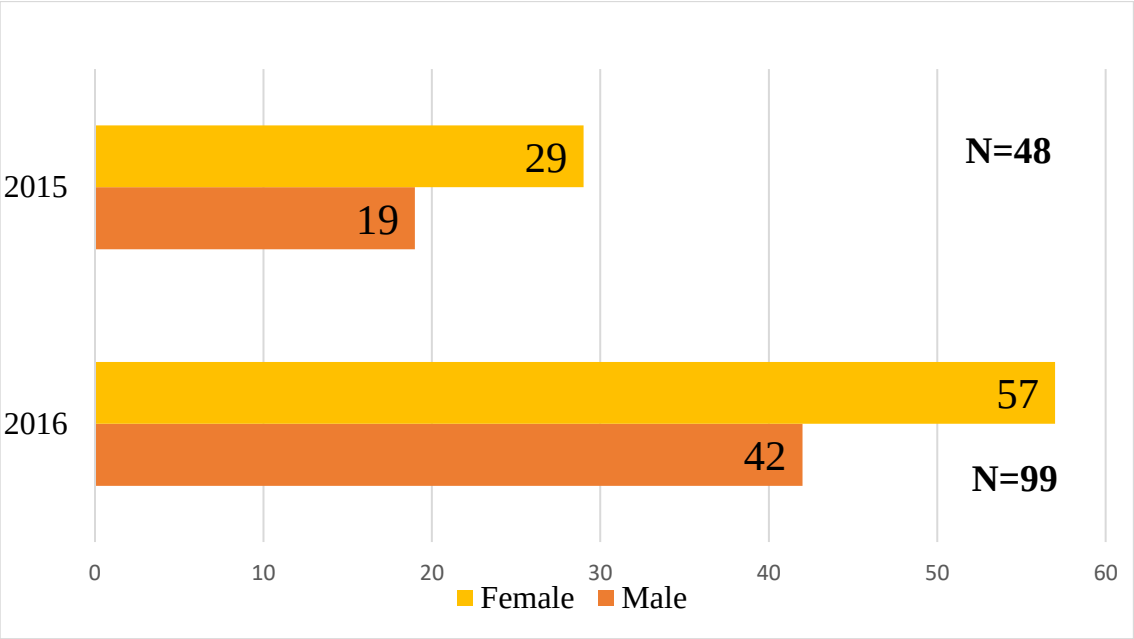


Figure 3.16 *C. difficile* infection according to gender in 2015 and 2016

Age of *C. difficile* active patients is contrasted in figure 3.17 for 2015 and 2016.

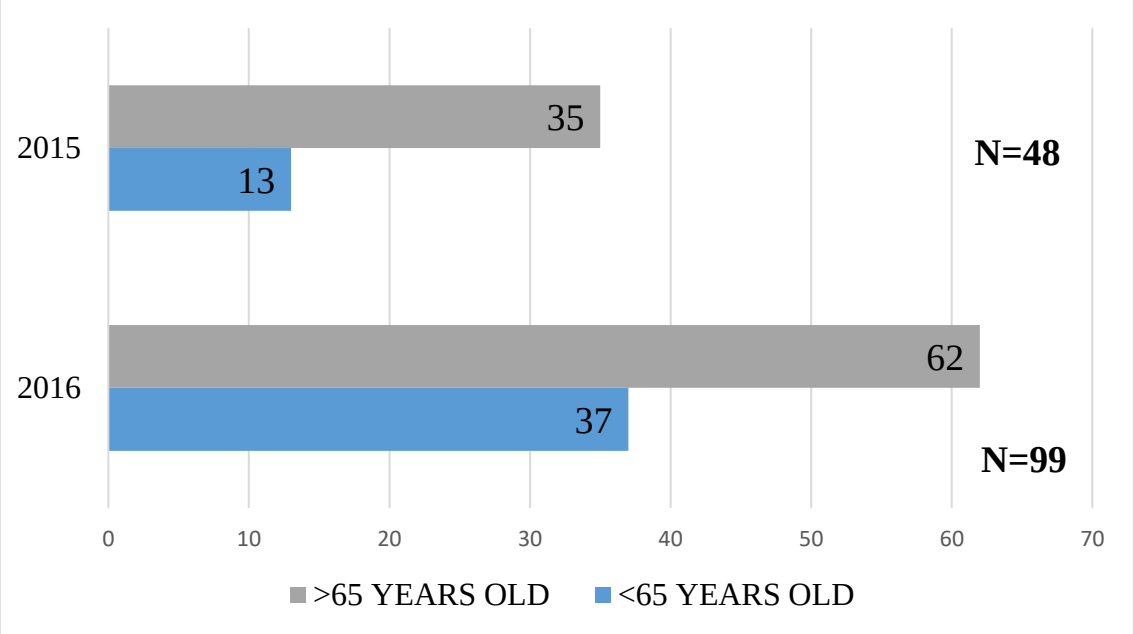


Figure 3.17 *C. difficile* infection according to age in 2015 and 2016

The difference of two proportions tests showed no statistical significance between male and female gender as a risk factor to acquire CDI in 2015 ($X^2(1) = 2.088$, p-value: 0.1484). The same test was performed for the 2016 gender data. No statistical significance was found ($X^2(1) = 2.266$, p-value: 0.1323) but data show a prevalence 1.5 times greater in the female population.

Table 3.20 Results from the two-proportion test for gender in 2015 and 2016

2015		**2016**	
Difference	0.0047%	**Difference**	0.00704%
95% Cl	-0.0020 to 0.0116	**95% Cl**	-0.0025 to 0.0167
Chi-squared	2.088	**Chi-squared**	2.266
DF	1	**DF**	1
Significance level	P=0.1484	**Significance level**	P=0.1323

The difference of two proportions test was subsequently applied to analyse the significance of the age as a risk factor to acquire CDI in 2015 and 2016. Statistical significance was found in 2015 ($X^2(1) = 112.362$, p-value: <0.0001) and in 2016 ($X^2(1) = 154.116$, p-value: <0.0001).

Table 3.21 Results from the two-proportion test for age in 2015 and 2016

2015		**2016**	
Difference	0.0465%	**Difference**	0.07839%
95% Cl	0.0311 to 0.0661	**95% Cl**	0.0573 to 0.1036
Chi-squared	112.362	**Chi-squared**	154.116
DF	1	**DF**	1
Significance level	P=<0.0001	**Significance level**	P=<0.0001

Chapter 4

Discussion

4.1 *Clostridium difficile* in clinical practice

C. difficile is the most common cause of hospital-acquired diarrhoea. There is evidence of variation in the *C. difficile* epidemiology with an increase in incidence due to the emergence of a more virulent strain causing treatment failure and an approximately four-fold increase in mortality (Deneve *et al,* 2009; Cohen *et al,* 2010; Musgrave *et al,* 2011; Huang *et al,* 2015; Shields *et al,* 2015)

The presence of these new resistant isolates highlights the need for new techniques to identify these strains and ensure appropriate treatment in the clinical setting. Culturing and antibiotic sensitivity testing from stool samples has been presented as an attractive method with economic viability to identify potential resistances and ensure suitable management practice. The standard procedure for *C. difficile* culturing and antibiotic sensitivity testing (Appendix 8) proposed in this study is not intended to serve as a diagnostic method but as an aid in *C. difficile* management once it has been identified. It has been elaborated for a practice clinical setting.

In this study estimates were calculated for 30 samples for an initial pilot study. An increase in purchase volumes will possibly involve a reduction in individual prices, providing a more attractive final quotation should culturing and antibiotic sensitivity testing be implemented within a clinical setting. When comparing costs, *C. difficile* culturing and antibiotic sensitivity testing shows a cost of €161, whereas the cost of stay in hospital is €175 per day and the treatment for CDI ranges from €7.50 (10 days of treatment with metronidazole) to €326.98/392.28 per treatment when metronidazole (intravenously) and vancomycin (orally) are administered together. This last amount represents three times the sum needed to run the culturing and antibiotic sensitivity testing according to the standard suggested procedure in this research. This demonstrates the

economic feasibility and sustainability of proposing this procedure so as to optimize better pharmacotherapy.

The recurrence rate in Malta is low (3 cases in 2015 and 6 cases in 2016 in the total population). This fact can be due to three reasons: The first is loss of follow-up of patients upon discharge not returning to the public health sector on recurrent presentation of symptoms. Another reason could be the lack of adherence to the local algorithm for *C. difficile* screening resulting in underdetection due to unnoticed/underestimated cases of CDI. The reliability of the current screening test could be the third factor influencing the low local recurrence rate.

In Malta, primary healthcare is provided by the private sector and the state. These two systems work independently of one another. It is estimated that 65% of the total healthcare expenditure is financed by the Government, leaving a burden to the private sector (Azzopardi *et al*, 2012). The absence of data sharing between these two entities obstructs follow-up of patients' progress.

It is hard to determine the number of symptomatic patients that are not screened for *C. difficile*, some of them being misdiagnosed. Overlooking some symptoms leads to diagnosis failure. This demonstrates that healthcare professionals' awareness about CDI is essential to identify the maximum number of cases.

Upon implementation of the local algorithm in the general hospital in April 2016, there has been a double-fold increase in the number of positive cases. The previous screening method involved identification of A & B toxins by EIA as a unique step. The new screening method includes a previous GDH EIA screening test followed by an A & B toxin EIA as a confirmatory test for GDH positive cases. This switch in methodology could have decreased the detection rate due to false negatives in the GDH EIA screening

test but the results show an increase. The reported cases with mixed results for 2 patients (continuous tests performance with different results) may suggest a potential low reliability in the current screening method adopted at MDH (initial screening for GDH antigen followed by a confirmatory toxin test for GDH positive results). Due to these considerations, the actual recurrence rate could be higher than detected.

Fluoroquinolones are used first line in the treatment of infectious diarrhoea. Fluoroquinolones are effective against *Salmonella, Shigella* and *Campylobacter*. New strains of *C. difficile* show resistance to fluoroquinolones. Fluoroquinolones use has emerged as a relevant risk factor for CDI.

Culturing and antibiotic sensitivity testing is performed in Scotland together with ribotyping methods as part of an epidemiological surveillance program (Cowden *et al,* 2008). A similar surveillance program was carried out in Spain between 2007 and 2011 (Webber *et al,* 2013) to provide data about susceptibility profiles and epidemiology of *C. difficile.* British guidelines recommend freezing of toxin positive stool samples to allow a retrospective culture in case of outbreaks of changes in the local epidemiology to monitor antimicrobial susceptibility. These guidelines additionally suggest culturing and antibiotic sensitivity testing of toxin positive samples as part of the Department of Health/Health protection agency surveillance program (Department of Health and Health Protection Agency, 2012).

Resistance to metronidazole and vancomycin has been reported in clinical isolates with poor clinical outcome (Barbut *et al,* 1999; Brazier *et al,* 2001; Pelaez *et al,* 2002; Fernandez *et al,* 2004; Musher *et al,* 2005; Pelaez *et al,* 2005; Warny *et al,* 2005). This highlights the need for frequent monitoring of potential emergence of drug resistance.

After data has been analysed and the local scenario has been assessed, *C. difficile* culturing and antibiotic sensitivity testing is suggested to be performed locally in recurrent cases in view of the potential presence of a resistant strain. A reflection from this study is that culturing and antibiotic sensitivity testing is proposed to be performed in cases of *C. difficile* outbreaks and in immununocompromised patients due to the need for prompt resistance identification and an initial accurate treatment. Culturing and antibiotic sensitivity testing is an essential tool for further research of local strains and maybe useful to establish an epidemiological surveillance program for CDI locally.

The total number of patients enrolled for this phase of the study was reduced due to the following reasons: transfer of the patient to a different hospital or private clinic, discharge of the patient or decease of the patient. Two patients that initially met the inclusion criteria and signed the written informed consent, were excluded due to mixed results issued from the virology laboratory. Faecal specimens were sent for *C. difficile* screening up to four times in the same week for each patient. These patients were reported as carriers of non-toxigenic and toxigenic *C. difficile* during the same week. This may suggest a low reliability of the screening test, innapropriate handling of the faecal sample or innacurate performance of the screening test.

The mean age was 63 years for the carrier population and 59 years for the active population, suggesting that older age is not a determinant factor to be a carrier or an active patient. This is supported by the Chi-square test which did not find the age as a significant factor of being a carrier or active patient. The influence of race as a risk to acquire CDI could not be determined due to the lack of heterogeneous population. Ninety-six percent of the studied population was White Caucasian race.

Penicillins and penicillin combinations were the most commonly associated antibiotics, reported in twenty-four cases in *C. difficile* carriers and in eleven occasions in *C. difficile* active patients. According to literature, clindamycin is the major predisposing antibiotic for CDI, followed by the group of penicillins as the second most frequently implicated agents for CDI (Keighley, 1980; Bartlett, 1981; Gilligan *et al,* 1981; Hirschhorn *et al,* 1994; Barlett, 2008). In this study, exposure to clindamycin antibiotic was reported in three *C. difficile* carrier patients and in four *C. difficile* active patients. Fluoroquinolones have recently been reported as risk factors for CDI (Mayhew, 2011). In this study, seven *C. difficile* carrier patients and eight *C. difficile* active patients had a recent exposure to fluoroquinolones. In this study, penicillins were the most prevalent group of antibiotics for both populations and not clindamycin as reported in literature. In literature, *C. difficile* active patients are the studied population and there is no clear evidence for the association between asymptomatic carriage of *C. difficile* and previous antibiotic exposure (Guerrero *et al,* 2013).

The onset of the infection was predominantly present in a health care facility for *C. difficile* carriers and actives. There are cases in the carrier population (14 out of 42 patients) and in the active population (7 out of 23 patients) that acquired the infection in the community setting. Neuberger *et al*, reported that CDI was more frequently acquired in the community setting by younger populations with previous empirical exposure to fluoroquinolones (Neuberger *et al,* 2013). Age of the population with community onset and health care facility onset of the infection was compared. Population with a community onset of the infection showed a mean age of 67 years and population with a health facility onset of the infection showed a mean age of 51 years. There was younger population on the group who acquired the infection in the community. These numbers suggest a higher risk for the younger population to acquire the infection from the community setting and

a greater risk for the elderly population to acquire the same infection on a health care facility setting. Comparison with a healthy group is needed to establish this correlation.

The length of stay in hospital was not a notable risk factor for the studied population to acquire the infection. Seventy-nine percent of the *C. difficile* carriers and 58% of the actives with a health care facility onset of the infection showed a length of hospital stay inferior to 20 days.

There was a low percentage in the affected population who were on probiotics, 9.5% of the *C. difficile* carriers and 17% of the *C. difficile* active patients. These patients were symptomatic. The use of probiotics in CDI has been of interest in the past years (Musgrave *et al,* 2011; Vincent *et al,* 2015). They have shown to cause "colonization resistance", increasing defense against *C. difficile* colonization. Probiotics can also play a role in preventing dysbiosis, which is a disruption of the gut flora caused by antibiotic use and which predisposes to CDI. Due to low available data of affected patients on probiotics, it was not feasible to determine their adequacy in CDI. Further research in this field is suggested in view of the potential beneficial role of probiotics as prophylaxic agents for this infection with a positive safety profile. The inclusion of probiotics in the local treatment algorithm as an adjuvant therapy is suggested since benefits are considered to outweigh the risks in this case.

Gastric acid plays a role as a bactericidal and toxin-neutralising agent. PPIs and H_2-receptor antagonists cause gastric acid suppression, lowering the resistance for *C. difficile* colonization. Gastric acid suppression is not confirmed as a risk factor for CDI but due to the evidence reported by recent publications, the FDA issued an announcement regarding this possible connection and suggested discontinuation of the gastric acid suppression when viable (Food and Drug Administration, 2012). This study shows a high prevalence

of gastric acid suppression treatment among the affected population (71% in *C. difficile* carrier patients and 74% in *C. difficile* active patients). Omeprazole is one of the most prescribed drugs in the world (Li *et al,* 2013). A sample of healthy population is needed to allow comparison with the affected group and determine the association between PPI therapy and CDI. Continous re-assessment of the need for gastric acid suppression therapy is proposed to decrease incidence of this infection.

Gastrointestinal perturbations such as gastrointestinal surgery and NGT feeding have been reported as risk factors for CDI. There was a high prevalence of gastrointestinal perturbation procedures in the affected population, being larger for the *C. difficile* active patients (57%). This results suggest routine monitoring of this group of patients for prompt detection and treatment to improve outcomes.

The reason for admission of the studied population does not follow a pattern and therefore no correlation was found. Immunosuppression has been linked to CDI. It was reported in 38% of the carrier populations and 39% of the active population. It was not considered a relevant risk factor in the affected population.

Chronic therapy was studied and it was found that PPIs (60%) followed by ACE inhibitors (31%) and high-ceiling diuretics (29%) were the most prevalent chronic therapies among the *C. difficile* carriers, whilst PPIs (74%), lipid-modifying agents (43%) and antithrombotic agents (39%) were more prevalent among *C. difficile* actives. Comparison with a healthy population is required to assess the statistical significance of these factors to acquire CDI. A review in relation to PPI overprescribing, especially at high dose or for long term in patients not a high risk of gastric damage maybe seen as a signal to be followed up from this study.

The adherence to the local algorithm was assessed by recording: testing adequacy, treatment according to algorithm, isolation of the patient, stool charting and discontinuation of non-clostridial antibiotics, antimotility agents and gastric acid suppression when possible. The patients were located in different wards all over the hospital. This fact is suggested as a potential factor responsible for the lack of adherence to the algorithm. As a recommendation from this study, relocation of these patients once *C. difficile* is detected to the IDU is advised to ensure optimal management of the condition. The delivery of educational programs to all the personnel involved in the patient's care in hospital is recommended to increase adherence to the local algorithm. The selection of the treatment was inappropriate in nine occasions. In two instances vancomycin was administered IV instead of orally. Vancomycin is administered orally in this infection to achieve local effect, involving minimal systemic absorption and lower side effects. When vancomycin is administered IV, there is a greater risk for nephrotoxicity, ototoxicity and the need for drug monitoring. Consequently, it is important to select the proper route when administering vancomycin for CDI. In one of the cases, omeprazole and domperidone were the selected treatment for this infection. This is an unsuitable therapy and PPIs are recommended to be suspended when CDI is present. A fluoroquinolone (ciprofloxacin) was selected in two occasions. Empiric use of fluoroquinolones has been reported as a risk factor for CDI, with ciprofloxacin being of relevance since it is a common treatment for infectious diarrhoea.

The continuation of the gastric acid suppression treatment without reported indication is a general issue. Inappropriate PPI use may increase the risk for community-acquired pneumonia, hip fractures, severe hypomagnesemia as well as CDI (Wick, 2016). Prolonged use of H_2-receptor antagonists can increase the risk of growing

enterochromaffin-like cell hyperplasia, which could result in gastric malignancy (Sabesin, 1993). H_2-receptor antagonists have been linked to non-small cell lung cancer in diabetic patients and the FDA is also reviewing the risk of CDI in users of histamine H_2 receptor blockers (Food and Drug Administration, 2012; Hsu *et al,* 2013). It is suggested the development of a program to reduce gastric acid suppression overprescribing by implementing routine assessments to these patients to reduce the risks associated to long term gastric acid suppression treatment in populations that no longer require this therapy.

In nine instances the *C. difficile* screening test result was not reported on the doctor's notes. This can be a result of the absence of symptoms when the result was issued. The isolation of the patient was reported in 75% of the cases. The non-isolated patients were asymptomatic. It is advisable to report the presence of *C. difficile* despite the patient being asymptomatic. This will allow the implementation of preventive measures to reduce contamination and spread of bacteria within the health care facility.

The factors involved in a higher or lower predisposition to develop the *C. difficile* active infection rather than carriage following *C. difficile* colonization were evaluated and statistically analysed using the Chi-square test. The factors that did not show to lead to a greater or lower proneness to acquire the active infection rather than the carriage after the statistical analysis were: older age, gender, onset of the infection, use of probiotics, gastric acid suppression, GI perturbations and immunosuppression. These factors do not determine whether the infection is going to progress to an active infection or remain asymptomatic. Recent antibiotic exposure and chronic kidney disease showed to increase the risk to progress to the active infection rather than the carriage. Recent antibiotic exposure was the only factor that exhibited statistical significance, but the logistic regression model also identified chronic kidney disease as a predictor of the outcome.

The Chi-square test provided a p-value of 0.087 for chronic kidney disease as a risk factor to progress to a *C. difficile* active infection rather remain as asymptomatic. This factor was considered significant since it was very close to the level of significance (0.05). This result evidence the need for monitoring those patients who suffer from chronic kidney disease and with GDH positive test result and toxin test negative result in view of the likely event to progress to *C. difficile* active infection.

The odds ratio for recent antibiotic exposure indicated that for a patient who was recently exposed to antibiotics, the odds that this patient is *C. difficile* active rather than carrier is 7.43 times that of a patient who was not exposed to antibiotics and the odds for chronic kidney disease indicated that the odds that a patient with this condition progresses to active rather than carrier is 3.81 times that of a patient who does not suffer from this condition.

This is an innovative study since *C. difficile* carriers and actives were not compared in these terms previously. This research shows evidence that a patient who has been colonized with *C. difficile,* had a recent antibiotic exposure and suffers from chronic kidney disease has a 94.1% of chance to be a *C. difficile* active rather than a carrier, indicating that these two factors are determinant to progress to the active infection. This result is in constrast to a patient who did not have a recent antibiotic exposure and does not present chronic kidney disease. In this case, the chances that this patient remains a carrier are 63.9%.

A patient who had a recent antibiotic exposure, suffers from chronic kidney disease and whose faecal specimen is positive for GDH antigen test but negative for the toxin test, is recommended to be closely monitored due to the high risk (94.1% of chances) to develop into the active infection according to the results presented in this study.

For the epidemiological study, information such as, gender, age and toxin test result was gathered. The comparison of *C. difficile* carriers from 2015 and 2016 was not achievable considering that GDH antigen test was not being performed in 2015 and the first three months of 2016. Toxin positive results from both years were correlated. The number of faecal specimens tested shows a two-fold increase. This can be explained by a greater awareness among the health care providers after the publication of the algorithm for CDI investigation and results interpretation in adults, which shows that educational programs would have an impact at detecting a greater number of cases. This is evidenced by the two-fold increase in toxin test positive samples and two-fold increase in the detection of *C. difficile* active patients. The number of recurrent cases increased by more than three times.

Gender of *C. difficile* active patients in Malta was compared for 2015 and 2016. In both years, a higher prevalence on the female population is apparent, being 1.5 times greater than the male population. The difference of two-proportion calculator did not disclose statistical significance, reporting a p-value of 0.1484 for 2015 and p-value of 0.1323 for 2016. All the national population who accessed health care services locally through the National Health Service was included in this phase of the study. This was consequently considered a signal from the study, suggesting female gender as a potential risk factor for CDI in the Maltese population. More research is suggested in this field for subsequent years.

The age of all *C. difficile* active patients in Malta was compared for 2015 and 2016. A greater prevalence in the population over 65 years old is noted. The difference of two-proportion calculator identified statistical significance in 2015 and 2016, reporting a p-value of <0.0001 in both cases. This result give robustness to previous studies that described the older age as a risk factor for CDI (Thomas *et al*, 2003; Peled *et al*, 2007).

This is the first study that statistically analyses epidemiological data from CDI patients in Malta, establishes older age as a risk factor and suggests female gender as a potential risk factor for CDI in the Maltese population.

The reported and published data display an increase in incidence, severity and mortality for CDI. This study shows that the epidemiology of CDI is not alarming in Malta but preventive measures and management to tackle this health care issue should be implemented before its presenteation and with priority.

Fluoroquinolone empiric treatment was identified as a determinant contributor for CDI in previous publications (Mayhew, 2011). In the studied population, there was a fluoroquinolone empiric treatment in 23% of the cases which highligths the needs for reassessment of this empiric prescribing under the presence of diarrhoea.

This is the first study that compares risk factors in *C. difficile* carriers and actives simultaneously, describing recent antibiotic exposure and chronic kidney disease as determining factors to acquire the active infection rather than carriage. This is the first comprehensive epidemiological study in Malta for CDI.

4.2 Limitations of the study

This study was conducted on a small population (Maltese population) which renders further extrapolation to larger populations difficult. Faecal specimens are mostly analysed in the virology laboratory at MDH but private practice also provides *C. difficile* screening testing from faecal specimens which are sent abroad for testing. These samples from the private practice were not included.

C. difficile screening test have a turnaround time of 24 hours at MDH. There were instances when the result was issued and the patient had been transferred to a different hospital, discharged or deceased. These cases were excluded due to the lack of signed witten informed consent, incurring in loss of patients for the study (63 patients). Since data from healthy individuals was not gathered, comparison to affected population was not feasible and new risk factors for CDI could not be identified.

When the affected population in 2015 and 2016 was statistically analysed, the total Maltese population was taken into consideration for comparison. The total affected population did not include cases from the private practice.

4.3 Further research

A pilot study for *C. difficile* culturing and antibiotic sensitivity testing in the clinical setting is proposed to assess its value for CDI management. More extensive studies comparing *C. difficile* active patients and carrier patients is suggested to identify futher risk factors to acquire the infection in both populations and to determine the role of chronic kidney disease as a risk factor to progress from *C. difficile* carriage to symptomatic. Additional studies with larger populations are suggested to establish the role of the gender as a risk factor for CDI. The importance of the exposure to penicillins

over clindamycin exposure as a predominant risk factor for CDI is proposed to be further studied in larger populations.

4.4 Conclusions

This study indicates that *C. difficile* culturing and antibiotic sensitivity testing should be performed locally in recurrent CDI, in case of *C. difficile* outbreaks, in immununocompromised patients, as a tool for further research of local strains and to establish an epidemiological surveillance program for CDI.

This is an innovative study that assesses risk factors for carriage of non-toxigenic *C. difficile* and toxigenic *C. difficile,* comparing these populations concomitantly. Recent antibiotic exposure and chronic kidney disease showed to increase the risk to progress to the active infection rather than the carriage. A regression model was developed and the results showed that for a patient who acquires CDI, there is a 7.43 more chance to progress to symptomatic infection when recent or concomitant antibiotic treatment and 3.81 more chance to progress to symptomatic infection when the patient suffers from chronic kidney disease. A patient who concomitantly suffers from chronic kidney disease and was recently exposed or is exposed to antibiotic treatment has 94.1% of chance to be a *C. difficile* active rather than a carrier.

There is need for the implementation of gastric acid suppression therapy routine assessment programs and a need to decrease empiric treatment with fluoroquinolones. Implementation measures to prevent contamination from *C. difficile* carriers are deemed necessary.

A higher prevalence in the female population was apparent, being as much as 1.5 times greater than the male population. Female gender is described as a potential risk factor for CDI among the Maltese population. It is a signal from the study that needs further investigation.

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Appendix 1

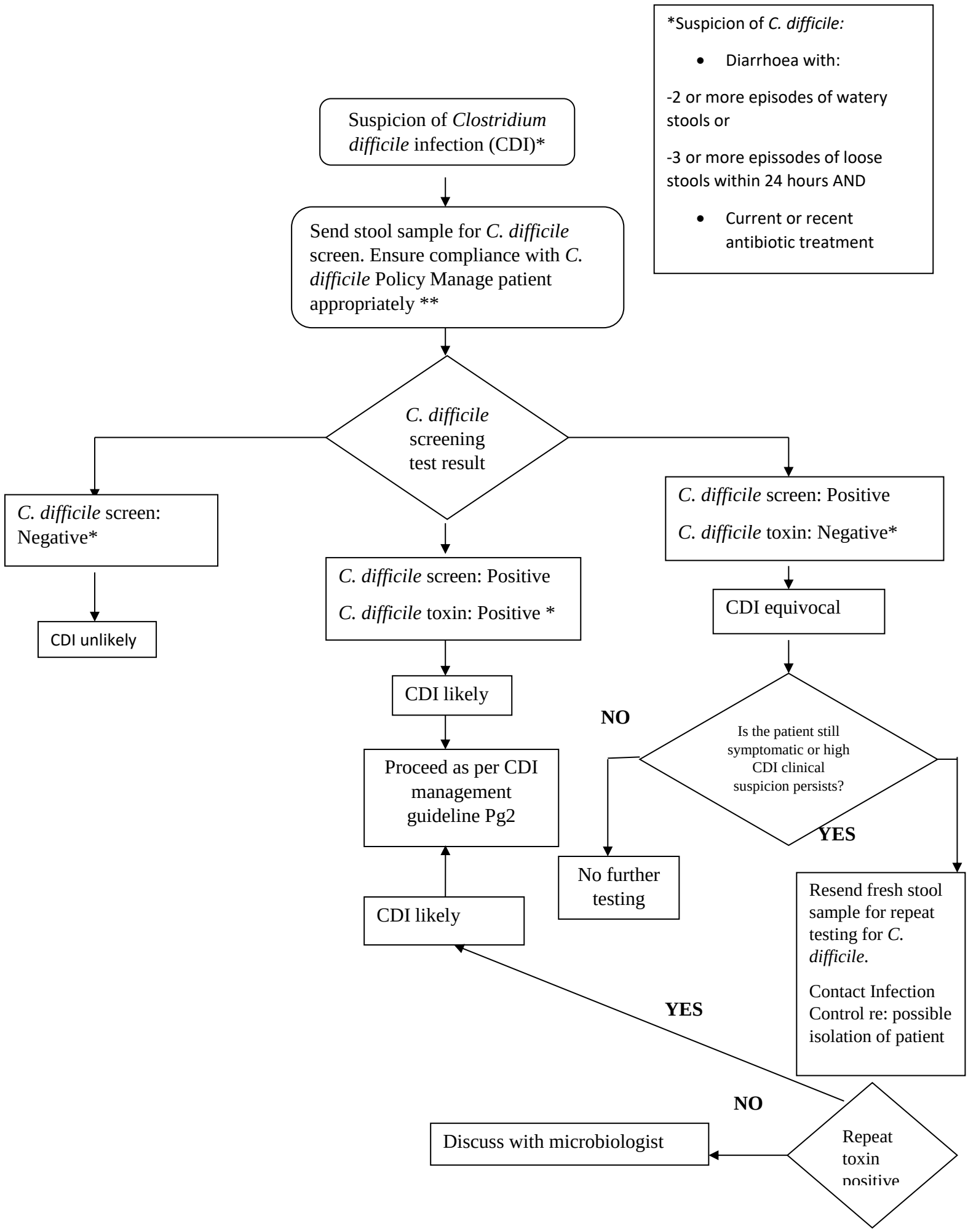
The Bristol Stool Form Scale (Bristol Stool Chart)

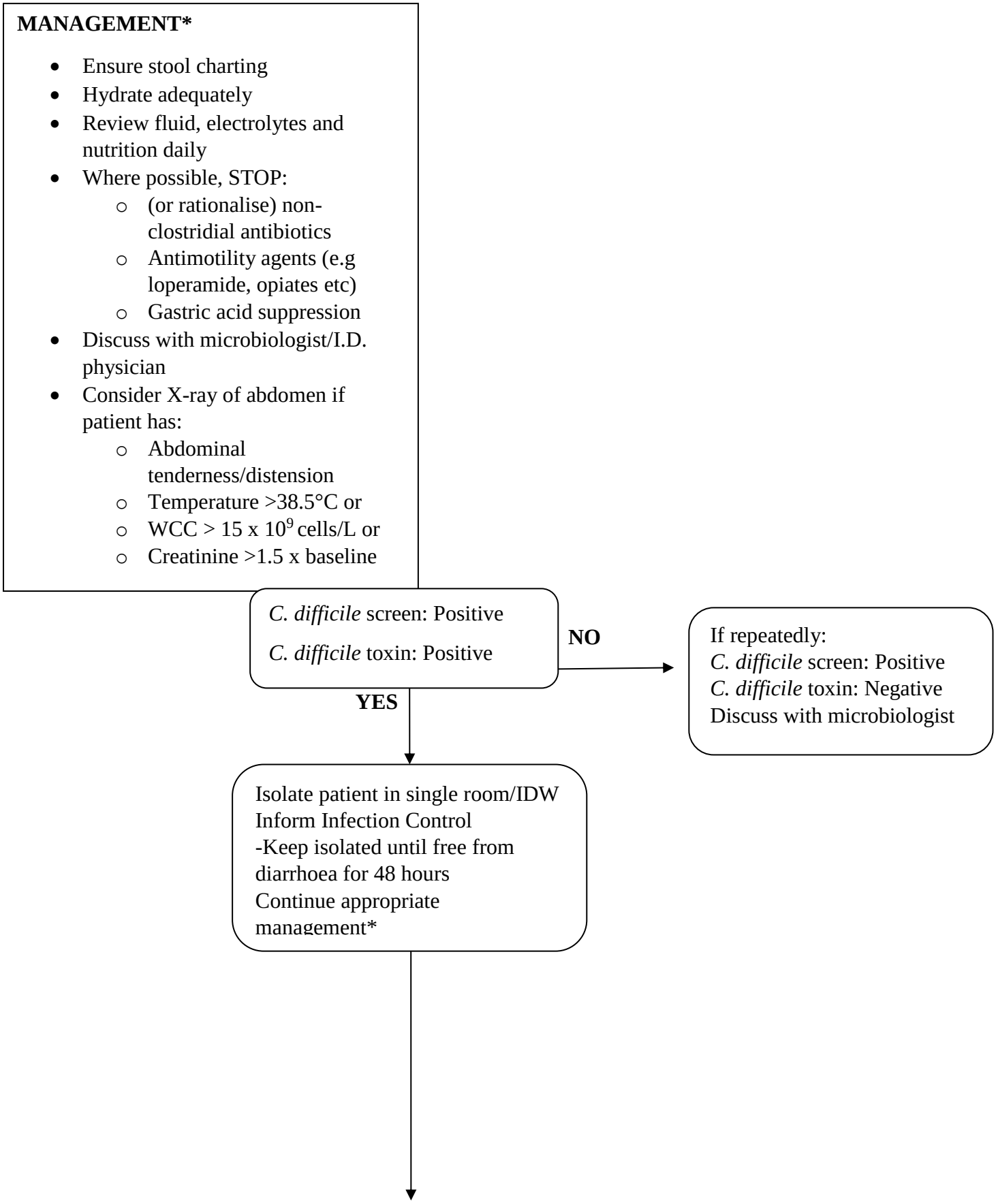
The Bristol Stool Form Scale (Bristol Stool Chart)

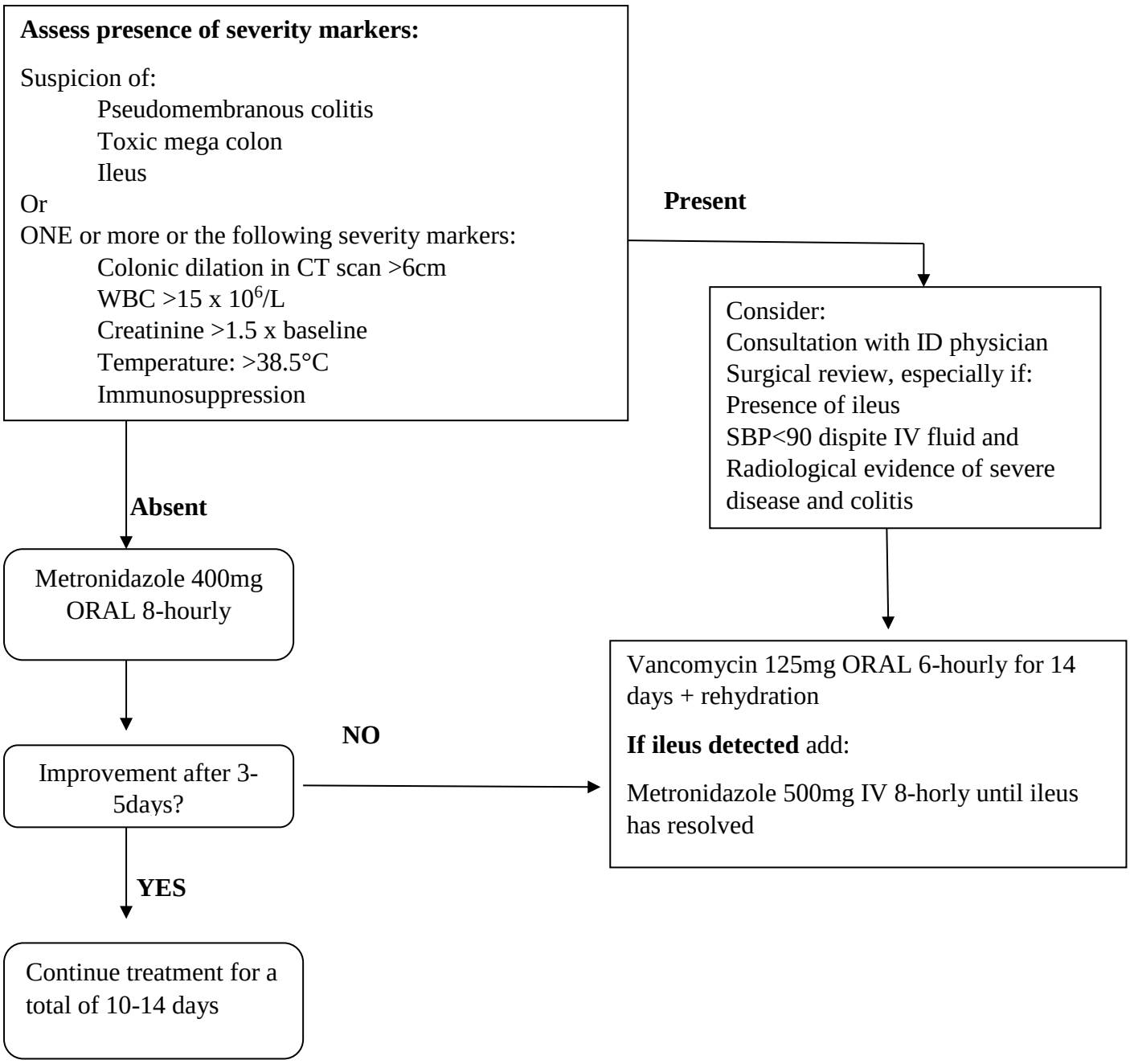
Type 1		Separate hard lumps, like nuts (hard to pass)
Type 2		Sausage-shaped but lumpy
Type 3		Like a sausage but with cracks on its surface
Type 4		Like a sausage or snake, smooth and soft
Type 5		Soft blobs with clear-cut edges (passed easily)
Type 6		Fluffy pieces, a mushy stool
Type 7		Watery, no solid pieces ENTIRELY LIQUID

Appendix 2:

Algorithm for *C. difficile* infection (CDI) investigation and results interpretation in adults


*Suspicion of *C. difficile:*
• Diarrhoea with:
-2 or more episodes of watery stools or
-3 or more episodes of loose stools within 24 hours AND
• Current or recent antibiotic treatment
Suspicion of *Clostridium difficile* infection (CDI)*
Send stool sample for *C. difficile* screen. Ensure compliance with *C. difficile* Policy Manage patient appropriately **
C. difficile screening test result
C. difficile screen: Negative*
CDI unlikely
C. difficile screen: Positive
C. difficile toxin: Positive *
CDI likely
Proceed as per CDI management guideline Pg2
C. difficile screen: Positive
C. difficile toxin: Negative*
CDI equivocal
Is the patient still symptomatic or high CDI clinical suspicion persists?
NO
No further testing
YES
Resend fresh stool sample for repeat testing for *C. difficile.*
Contact Infection Control re: possible isolation of patient
Repeat toxin positive
NO
Discuss with microbiologist
YES
CDI likely


MANAGEMENT*
Ensure stool charting
Hydrate adequately
Review fluid, electrolytes and nutrition daily
Where possible, STOP:
(or rationalise) non-clostridial antibiotics
Antimotility agents (e.g loperamide, opiates etc)
Gastric acid suppression
Discuss with microbiologist/I.D. physician
Consider X-ray of abdomen if patient has:
Abdominal tenderness/distension
Temperature >38.5°C or
WCC > 15 x 10^9 cells/L or
Creatinine >1.5 x baseline
C. difficile screen: Positive
C. difficile toxin: Positive
NO
If repeatedly:
C. difficile screen: Positive
C. difficile toxin: Negative
Discuss with microbiologist
YES
Isolate patient in single room/IDW
Inform Infection Control
-Keep isolated until free from diarrhoea for 48 hours
Continue appropriate management*


Assess presence of severity markers:
Suspicion of:
Pseudomembranous colitis
Toxic mega colon
Ileus
Or
ONE or more or the following severity markers:
Colonic dilation in CT scan >6cm
WBC >15 x 10^6/L
Creatinine >1.5 x baseline
Temperature: >38.5°C
Immunosuppression
Present
Consider:
Consultation with ID physician
Surgical review, especially if:
Presence of ileus
SBP<90 dispite IV fluid and
Radiological evidence of severe disease and colitis
Absent
Metronidazole 400mg ORAL 8-hourly
Improvement after 3-5days?
NO
Vancomycin 125mg ORAL 6-hourly for 14 days + rehydration
If ileus detected add:
Metronidazole 500mg IV 8-horly until ileus has resolved
YES
Continue treatment for a total of 10-14 days

C. difficile infection (CDI) Testing Guidance and Result interpretation in adults

1. Patients with loose stools should be isolated to prevent the transmission of *C. difficile,* norovirus or other transmissible pathogens, as per Gastroenteritis Infection control policy.
2. Only diarrhoeal stools will be tested for *C.difficile*. The stool sample must take on the shape of the container and ideally be at least ¼ filled. If a patient has diarrhoea (Bristol stool types 5-7) that is not clearly attributable to an underlying condition (e.g. inflammatory colitis, overflow) or therapy (e.g laxatives, enteral feeding) then it is necessary to determine if this is due to CDI. Stools from all such symptomatic patients should be collected as early as possible, for diagnostic and infection control purposes.
3. *C.difficile* toxin tests are not suitable as stand alone tests for the diagnosis of CDI thus a two-stage testing approach is to be undertaken. This consists of an initial sensitive screening test for Glutamate Dehydrogenase antigen of C.difficile (GDH), followed by a confirmatory toxin test for GDH positive cases.

   GDH test detects GDH antigen that is produced in high amounts by C.difficile, both toxin and non-producing strains.

   CDI is a toxin mediated disease thus the toxin test is used to detect the presence of C.difficile toxin/s that are specific for CDI including C.difficile colitis or pseudomembranous colitis.
4. The testing algorithm combines optimised performance with the ability to clinically categorise patients into one of three groups:

   a. If GDH antigen positive and toxin positive; this is reported as 'C.difficile screen and C.difficile toxin positive. CDI is likely.

   b. If GDH antigen positive and toxin negative, this is reported as 'C.difficile screen positive, C.difficile toxin negative.CDI Equivocal' result. C.difficile GDH antigen

present in the absence of toxin often reflects colonisation rather than infection; C.difficile excretion and transmission is possible. Clinical assessment is needed with consideration for repeat toxin testing on a fresh sample if the patient is still symptomatic or if high clinical suspicion of C.difficile remains. Infection control precautions, including patient isolation or cohorting are to be adhered to till 48hours diarrhoea free; liaison with Infection Control Unit if needed.

A second equivocal result, on repeat toxin testing in the same patient is again suggestive of colonisation rather than infection, with a retained potential for transmission.

Clinical assessment is needed; consider discussion with microbiologist or ID physician for further advice and consider CDI treatment if a high clinical suspicion of CDI remains.

Infection control precautions including isolation are to be adhered to till the patient is diarrhoea free for 48hours.

c. If GDH antigen negative. Reported as '*Clostridium difficile* screen negative. CDI unlikely.' If symptoms persist and no alternate diagnosis is found, consider sending a further sample for C.difficile testing

Note that toxin testing is not performed on C.difficile GDH negative specimens.

5. No test or combination of tests is infallible and the clinical condition of the patient should always be taken into consideration when making management choices. It should always be remembered that diagnosis of CDI is based on both the clinical presentation and the results of any laboratory tests, and that suspected C.difficile should be treated as per severity criteria (refer to guideline on page 2). Patients may occasionally require treatment for presumptive CDI before test results are available if signs/symptoms indicate infection.

6. In suspected cases of ‘silent CDI’ such as ileus, toxic megacolon or pseudomembranous colitis without diarrhoea, other diagnostic procedures, such as colonoscopy, white cell count (WCC), serum creatinine and abdominal computerised tomography (CT) scanning may be required.
7. Clearance testing is not recommended. Individuals can remain toxin positive for some weeks after symptoms have settled.
8. Repeat testing in confirmed positive cases should only be undertaken where symptoms have recurred after initial successful treatment. Repeat sampling in confirmed positive cases should NOT be performed within 10 days of a positive sample.

Reference: UK Standards for Microbiology Investigations B 10: Processing of faeces for *Clostridium difficile*.

Appendix 3

Form C: Case-based data (light and enhanced surveillance)

European surveillance of *Clostridium difficile* infections.
Form C: Case-based data (light and enhanced surveillance)

Hospital code: ___MT001_____

Surveillance period: From _01__ / _01__ / 2016___ (dd/mm/yyyy) to: _31__ / _12__ / 2016___ (dd/mm/yyyy)

Patient counter:

_____

ID number (Internal patient code):_____

Name of patient

_____

Sex:

- ☐ Male
- ☐ Female

Age in years: ____; age if < 2 years old: ____ months.

Previous healthcare admission in the last 3 months (optional)**:**

- ☐ Yes
- ☐ No
- ☐ Unknown

If yes, please specify:

- ☐ Hospital
- ☐ Long-term care facility
- ☐ Other (e.g. out patients)

Date of hospital admission: ___ / ___ / 20___ (dd/mm/yyyy)

Ward/unit ID (optional)**:** _____

Ward/unit specialty (optional; see code list): _____

Ward/unit name (optional)**:**_____

Patient/Consultant specialty (see code list): _____

McCabe score *(optional)**:***

- ☐ *Non-fatal underlying disease (survival at least 5 years)*
- ☐ *Ultimately fatal underlying disease (survival 1–4 years)*
- ☐ *Rapidly fatal underlying disease (survival <1 year)*
- ***X*** ***Unknown***

Symptoms of CDI present at admission:

- ☐ Yes
- ☐ No
- ☐ Unknown

If **NO: Date of onset of CDI symptoms:**__ / ___ / 20___ (dd/mm/yyyy)

Date of first positive sample (optional)**:** ___ / ___ / 20___ (dd/mm/yyyy)

Recurrent CDI (positive laboratory tests for CDI in diarrhoeal stools after the end of treatment for CDI occurring > 2 weeks and < 8 weeks following the onset of a previous episode):

- ☐ Yes
- ☐ No
- ☐ Unknown

European surveillance of *Clostridium difficile* infections. Form C: Case-based data (light and enhanced surveillance) - continued

CDI case origin (*tick one*):

☐ **Healthcare-associated** (symptom onset on day three or later following admission to a healthcare facility on day one, OR in the community within 4 weeks following discharge from any healthcare facility)
If yes, please specify:

- ☐ Current hospital
- ☐ Other hospital
- ☐ Long-term care facility
- ☐ Other healthcare facility (e.g. outpatient)

☐ **Community-associated** (symptom onset [outside of healthcare facilities, AND without discharge from a healthcare facility within the previous 12 weeks], OR [on the day of admission to a healthcare facility or on the following day AND no residence in a healthcare facility within the previous 12 weeks])

☐ **Unknown association** (including cases discharged from a healthcare facility 4–12 weeks before symptom onset)

Complicated course of CDI (optional)**:** (e.g. admission to a healthcare facility for treatment of a community-associated CDI; CDI resulted in e.g. ICU admission, toxic megacolon, surgery or death)

- ☐ Yes
- ☐ No
- ☐ Unknown

Patient outcome *(tick one)*:

- ☐ Discharged alive
- ☐ Death, CDI definitely contributed to death
- ☐ Death, CDI possibly contributed to death
- ☐ Death, no relation to CDI
- ☐ Death, relationship to CDI unknown
- ☐ Unknown

Date of hospital discharge/in-hospital death: ___ / ___ / 20_____(dd/mm/yyyy)

Microbiological data (Form M) collected for this patient:

- ☐ Yes
- X No
- ☐ Unknown

Appendix 4

University Research Ethics Committee approval

L-UNIVERSITÀ TA' MALTA

Msida – Malta
Skola Medika
Sptar Mater Dei

UNIVERSITY OF MALTA

Msida – Malta
Medical School
Mater Dei Hospital

Ref No: **20/2016**

Monday 8th August 2016

Ms. Noelia Holgado Sanchez
67, Thornfield House
Triq il-Mithna
Attard

Dear Ms. Noelia Holgado Sanchez,

Please refer to your application submitted to the Research Ethics Committee in connection with your research entitled:

Pharmacotherapy in the treatment of clostridium difficile: Impact on Clinical Practice

The University Research Ethics Committee granted ethical approval for the above mentioned protocol.

Yours sincerely,

Appendix 5

Data collection sheet

Patient name		**Date of admission**	
ID card number		**Date of data collection**	
Firm		**Reason for admission**	
Ward		**Date of diarrhoea presentation**	
Age		**Length of stay**	
Occupation		**Lab results**	**GDH (screen)**
Allergies			**Toxin**
Smoker/alcohol abuse			

Past medical history		
Past surgical history		
Chronic medication		
Medication initiated upon admission		
Medication stopped upon admission		
Previous use of antibiotics (last 3 months)	Aminoglycosides	Monobactams
	Carbapenems	Nitrofurans
	Cephalosporins 1st G	Oxazolidinones
	Cephalosporins 2nd G	Penicillins
	Cephalosporins 3rd G	Penicillin comb
	Cephalosporins 4th G	Polypeptides
	Glycopeptides	Quinolones
	Lincosamimdes	Sulfonamides
	Lipopeptide	Tetracyclines
	Macrolides	Others

Empiric treatment for CDI		
Bristol stool chart number		
Nasogastric tube		
Use of probiotics		
Use of PPIs		
Use of H_2-receptor antagonists		
Patient intubation		
Use of catheter		
Isolation		
Dialysis		
Test number		
CDI management	Metronidazole 400mg Orally	
	Vancomycin 125mg orally	
	Vancomycin 125mg orally + Metronidazole 500mg IV	

Appendix 6

Patient consent form English & Maltese

[English version]

Information consent form for _____

This informed consent form is for those patients gathering the characteristics needed to carry out a research named: "Pharmacotherapy in the treatment of *Clostridium difficile:* impact on clinical practice".

This informed consent has two parts:

1. **Information Sheet (to share information about the research with you)**
2. **Certificate of Consent (for signatures if you agree to take part)**

1. Information Sheet

My name is Noelia Holgado Sanchez, I am a Pharm D student at the University of Malta currently carrying out a research named: "Pharmacotherapy in the treatment of *Clostridium difficile:* impact on clinical practice".

I am going to give you information and invite you to be part of this research. You do not have to decide today whether or not you will participate in the research. Before you decide, you can talk to anyone you feel comfortable with about the research.

There may be some words that you do not understand. Please ask me to stop as we go through the information and I will take time to explain. If you have questions later, you can ask me or the rest of the staff.

This is a research about an intestinal infection very commonly developed in patients with risk factors in hospital and its main symptom is diarrhoea.

This is an infection that must be detected as earliest as possible in order to start antibiotic treatment.

According to hospital protocol, collection of stool sample is needed to diagnose this infection.

Half of the collected sample will be allocated for this research while the remaining fraction will be for diagnostic purposes.

In the case of active infection, the fraction concerning this research will be analysed in the laboratory to check which antibiotic is the most effective.

Another sample will then be collected after 14-28 days from the end of the treatment against *Clostridium difficile* as per hospital protocol and will be tested for antibiotic effectiveness of the treatment in cases of persistent infection.

The aim of the study is to find a potentially more effective treatment against this infection.

The duration of the study is until the 31st of December 2016.

Personal and medical information will be gathered from medical records, including name of the patient in order to allow follow-up in case of recurrence.

Your participation in this research is entirely voluntary. It is your choice whether to participate or not. This research will not influence your current treatment. You are only giving the consent to make use of part of stool sample already being collected for diagnostic purposes, hereby no personal risks or benefits are involved.

Confidentially

The information that we collect from this research project will be kept confidential. We will assign a code for each patient to maintain confidentiality.

Right to refuse or withdraw

You have the right to refuse the partial use of your stool sample for the aim of this research at any time.

2.

a) Certificate of consent

I have read the foregoing information, or it has been read to me. I have had the opportunity to ask questions about it and any questions that I have asked have been answered to my satisfaction. I consent voluntarily to be a participant in this research.

Name of Participant (Block letters)

Signature of Participant _____

Date _____

b) Certificate of consent for guardians in case illiterates.

A literate witness must sign (if possible, this person should be selected by the participant and should have no connection to the research team).

I have witnessed the accurate reading of the consent form to the potential participant, and the individual has had the opportunity to ask questions. I confirm that the individual has given consent freely.

Print name of witness_____

Signature of witness _____

Date _____

Statement by the researcher/person taking consent

I have accurately read out the information sheet to the potential participant, and to the best of my ability made sure that the participant understands that the following will be done:

1. Stool sample(s) will be collected to carry out the research "Pharmacotherapy in the treatment of *Clostridium difficile:* impact on clinical practice" as per hospital protocol.

2. Patient's treatment will not be modified at any time.

3. Patient's detail will remain confidential.

I confirm that the participant was given an opportunity to ask questions about the study, and all the questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

A copy of this ICF has been provided to the participant.

Print Name of Researcher/person taking the consent_____

Signature of Researcher /person taking the consent_____

Date _____

Researcher contact details:

Noelia Holgado Sanchez

Tel: 99969481

email: noelia.holgado.14@um.edu.mt

[Maltese version]

Formola ta' kunsens ghal-pazzjent:- Sinjur/a _____

Din il-formola ta' kunsens hi indirizzata lil dawk il-pazzjenti li huma interessati jippartecipaw fi studju entitolat: “Pharmacotherapy in the treatment of *Clostridium difficile:* impact on clinical practice”.

(L-uzu tat-terapijja farmacewtika fl-itrattament tal-infezzjoni kkawzata mill-ispeci ta' batterja *Clostridium difficile*, kif ukoll l-impatt ta’ dan fuq il-prattika klinika.

Din il-formola ta' kunsens hi maqsuma f'zewg parti:

1. **It-taqsima informattiva (sabiex infehmuk precizament dwar dak li minnu ser tkun qieghda tikkonsisti din ir ricerka)**

2. **Certifikat tal-kunsens (sabiex tikkonferma ix-xewqa tieghek li tippartecipa f'dan l-istudju, permezz tal firma tieghek [2a] jew inkella tal-raprezentant tieghek [2b])**

1. It-taqsima informattiva:-

Gheziz/a Sinjur/a, insellimlek. Jiena jisimni Noelia Holgado Sanchez. L-okkupazzjoni tieghi hi ta' studenta fih dan l-Universita' ta' Malta, fejn qieghda nistudja ghal dottorat fil-farmacija imsejjah Pharm D. L-istudju li qieghda inhejji sabiex nibni tezi fuqu, jismu “Pharmacotherapy in the treatment of *Clostridium difficile:* impact on clinical practice”.

Nixtieq niehu din l-opportunita' biex ninfurmak dwar u inheggek tiehu sehem f' din ir-ricerka tant importanti ghalijja. Ma hemmx ghalfejn tasal ghal decizjoni dwar jekk tixtieq tippartecipa, minnufih. Biss, qabel ma' tiehu din id-decizjoni nistiednek titkellem u tiddiskuti ma' nies qrib tieghek, jew ma' min tixtieq; sabiex thossok cert/a minn din ir-ricerka.

Jista' jkun hemm ftit kliem tekniku li ma tifhimx, jien u nispjegalek dwar dan l-istudju. Jekk jaghti l-kas, nitolbok twaqqafni dak il hin stess sabiex niccaraw l-affarjiet minnufih. Jekk ikollok xi

mistoqsija, inheggek tindirizzahom lili jew lejn l-istaff mediku.

Dan l-istudju jitratta dwar infezzjoni komuni fil-musrana, li hi prevalenti f' certi pazzjenti li qieghdin jircievu kura l-isptar, peress li huma ghandom fatturi ta' riskju gholjin ghal mard bhal din. Is-sintomu ewlieni ta' din l-infezzjoni hi d-diarrhoea (l-ippurgar b' konsistenza likwida).

Jehtieg li t-trattament ghall-infezzjoni ikkoncernata jibda' minnufih u bikri kemm hu possibli, permezz ta' medicini imsejhin anti-bijotici.

Skond l-iprotocol tal-isptar, jehtieg l-uzu ta' kampjuni tal-ippurgar sabiex tigi ddeterminata l-prezenza ta' din l-infezzjoni.

Nofs il-kampjun migbur ser jigi allokat ghal din ir-ricerka, filwaqt li n-nofs li jibqa' ser jintuza ghal ragunijiet diagnostici.

Fil-kas ta' infezzjoni attiva, in-nofs tal-kampjun li gie allokat ghal din ir-ricerka ser jigi analizzat gewwa laboratorju, sabiex tigi ddeterminata liem kura (permezz tal-medicini anti-bijotici) hi l-aktar effettiva.

Kampjun iehor tal-ippurgar ser jittiehed 14-28 gurnata minn meta jintemm l-itrattament kontra *Clostridium difficile*, kif inhu imnizzel fil-protocol tal-isptar. Dan il-kampjun ser jigi ezaminat ghal effettivita' anti-bijotika tal-itrattament fil-kas ta' infezzjoni persistenti.

L-ghan ta' dan l-istudju hu biex tinsab l-aktar kura effettiva ghal din l-infezzjoni. L-istudju ser jintemm fil-31 ta' Dicembru, 2016.

Informazzjoni personali kif ukoll informazzjoni li titratta dwar sahhet il-partecipant (i.e. medika) ser jittiehdu mir-rekordi medici tal-istess partecipant. Informazzjoni personali kif ukoll informazzjoni li titratta dwar sahhet il-partecipant (i.e. medika) ser jittiehdu mir-rekordi medici tal-istess partecipant. L-informazzjoni migbura ser tinkludi l-isem u l-kunjom tal-pazzjent sabiex tkun tista' ssir *follow up* (ezaminazzjoni ohra) fil-kas li l-infezzjoni terga' titfacca

Il-partecipazzjoni tieghek ghal din ir-ricerka hi b'mod totalment voluntarju sa mill-ewwel minuta.

Ghaldaqstant, l-ghazla biex tiehu sehem hi f'idejk. Din ir-ricerka m'hix ser taffetwa jew tinfluwenza kwalinkwe trattament li qieghed/a tircievi bhalissa. Nixtieq infakkrek li l-kunsens tieghek jippermettilna niehdu u naghmlu uzu minn parti tan-nofs il-kampjun tal-ippurgar tieghek; i.e. in-nofs li diga' gie allokat ghal ragunijiet diagnostici; jigifieri mhux ser ikun hemm riskji kif ukoll beneficji involuti.

Kunfidenzjalita':

L-informazzjoni personali tieghek li ser nigbru ghal dan il-progett ta' studju ser jinzamm kunfidenzjali. Biex naccertaw dan u nserhulek mohhok, ser naghtuk kodici personali uniku (numru).

Id-dritt biex tirrifjuta jew twaqqaf is-sehem tieghek f'kwalinkwe stadju tal-istudju:

Ghandek id-dritt tirrifjuta l-alokazzjoni parzjali tal-kampjun tieghek ghal-ghan ta' din ir-ricerka, meta trid.

2a. Certifikat tal-kunsens:-

Jien, _____, nikkonferma li qrajt l-informazzjoni kollha, jew inkella giet moqrijja lili. Kelli l-opportunita' li nsaqsi mistoqsiejiet, u dawn kollha gew imwiegba b'mod sodisfacenti fl-opinjoni tieghi. Ghaldaqstant, naghti l-kunsens tieghi sabiex nippartecipa b'mod voluntarju f' din ir-ricerka.

Isem u kunjom tal-partecipant (f'ittri kbar jekk joghgbok): _____

_____

_____

Firma tal-partecipant: _____

Data: _____

2b. Certifikat tal-kunsens ghar-raprezentant/xhud, jekk il-partecipant hu/hi illitterat/a:-

Ix-xhud (li ghandu jkun litterat) hu mitlub jiffirma (din il-persuna ghandha tigi maghzula mil-partecipant u idealment ma jkollux/hix kuntatt man-nies li ser imexxu r-ricerka).

Jien, _____(ix-xhud) kont prezenti kif ukoll qrajt il-formola ta' kunsens ghal beneficju tal-partecipant potenzjali; u naccerta li dan tal-ahhar kellu/ha l-opportunita' i/ssaqsi mistoqsijiet. Nikkonferma li l-partecipant qed jaghti l-kunsens tieghu b'mod voluntarju.

Firma tax-xhud: _____

Data: _____

Stqarrija tar-ricerkatur jew il-persuna li ser tigbor il-kunsens minghand il-partecipant:-

Nikkonferma li qrajt l-informazzjoni kollha lil-partecipant potenzjali b'mod shih, preciz u car; u fil-fehma tieghi ghamilt dak kollu li stajt sabiex infihem lil-partecipant li l-punti li jmiss hawn isfel ser ikunu il-bazi tal-proceduri li ser isiru sabiex tingabar din ir-ricerka:-

1. Il-kampjun/i tal-ippurgar ser jittiehdu sabiex issir ir-ricerka entitolata "Pharmacotherapy in the treatment of *Clostridium difficile:* impact on clinical practice". (L-uzu ta' kampjuni tal-ippurgar sabiex tigi iddeterminata l-prezenza tal-ispeci ta' batterja, li isimha hu miktub bil-korsiv).
2. Din ir-ricerka m'hix ser taffetwa jew tinfluwenza kwalinkwe trattament li l-partecipant ikun qieghed jircievi/tircievi.

3. Id-dettalji tal-partecipant ser jibqghu kunfidenzjali.

Nikkonferma li l-partecipant inghata l-opportunita' biex jistaqsi kull domanda li xtaq rigwart l-istudju kkoncernat, kif ukoll li ghamilt dak kollu li stajt u fl-ahjar tal-abilta' tieghi biex inwiegeb dawn id-domandi kollha b'mod korrett.

Nikkonferma wkoll li l-partecipant ma giex imgieghel biex jaghti l-kunsens tieghu ghal din ir-ricerka, kif ukoll li l-kunsens li nghata mil-partecipant kien b'mod liberu u voluntarju.

Kopja ta' din il-formula tal-kunsens infurmat inghatat lil-partecipant.

Isem u kunjom tar-ricerkatur jew tal-persuna li qed tigbor il-kunsens: _____

_____

_____

Firma tar-ricerkatur jew tal-persuna li qed tigbor il-kunsens: _____

_____

Data: _____

Dettalji tar-ricerkatrici:-

Noelia Holgado Sanchez

Tel: 99969481

email: noelia.holgado.14@um.edu.mt

Appendix 7

Classification of patients' medication according to ATC classification system

Drug ATC classification	Drugs
ACE inhibitor	Perindopril
Adrenergic inhalant	Formoterol
	Salbutamol
Alpha-adrenoreceptor antagonist	Doxazosin
Angiotensin II antagonist	Valsartan
Antiarrhythmic	Amiodarone
Anti-dementia drug	Donepezil
Antidepressant	Amitriptyline
	Clomipramine
	Duloxetine
	Escitalopram
	Fluoxetine
	Mianserin
	Nortriptyline
	Oropram
	Paroxetine
Antidiarrheal	Loperamide
Antiemetic and antinauseant	Ondansetron
Antiepileptic	Clonazepam
	Levitaracetam
	Phenobarbital
	Phenytoin
	Pregabalin
	Sodium Valproate
Antifibrinolytic	Tranexamic acid
Antifungals for systemic use	Fluconazole
	Itraconazole
Antiglaucoma preparation	Latanoprost
	Timolol
Antigout preparation	Allopurinol
Antihistamine for systemic use	Chlorphenamine
	Promethazine
Antiinflamatory and antirheumatic product	Diclofenac
Antimetabolite	Azacitidine
	Cytarabine
Antineoplastic agent	Etoposide
	Doxorubicine
Antiparkinson drug	Ropinirole
Antipsychotic	Chlorpromazine
	Flupentixol
	Haloperidol
	Olanzapine
	Prochlorperazine
	Risperidone

Antithrombotic agent	Acetylsalicylic acid
	Clopidogrel
	Dipyridamole
	Enoxaparin
	Warfarin
Anxiolytic	Hydroxyzine
Beta blocking agents	Carvedilol
Biphosphonate (oral)	Alendronic acid
Blood glucose lowering drugs	Gliclazide
	Glimepiride
	Metformin
Calcium	Calcium carbonate
Capillary stabilizing agents	Diosmin combinations (Daflon®)
Cardiac glycosides	Digoxin
Contact laxative	Bisacodyl
Corticosteroid for systemic use	Dexamethasone
	Prednisolone
Cytotoxic antibiotics and related	Daunorubicin
	Idarubicin
Direct acting antiviral	Abacavir
	Acyclovir
	Efavirenz
	Lopinavir
	Ritonavir
Drug for functional gastrointestinal disorders, propulsive	Domperidone
	Metoclopramide
Drug for obstructive airway diseases, inhalant	Budesonide
	Fluticasone
	Ipratropium bromide
Drugs for peptic ulcer and gastro-oesophageal reflux disease (GORD)	Ranitidine
Drugs used in benign prostatic hypertrophy	Dutasteride
	Tamsulosine
Folic acid and derivatives	Folic acid
High-ceiling diuretics	Bumetanide
	Furosemide
Hormone antagonist and related agents	Tamoxifen
Hypnotics and sedatives	Bromazepam
	Diazepam
	Lorazepam
	Zolpidem
Intestinal antiinflamatory agents	Mesalazine
Immunostimulant	G-CSF
Immunosuppressant	Ciclosporin
	Infliximab

	Lenalidomide
	Methotrexate 20mg
	Mycophenolate acid
	Tacrolimus
Insulin and analogue for injection	Actrapid®
	Humilin M3®
Lipid modifying agents	Atorvastatin
	Bezafibrate
	Rosuvastatin
	Simvastatin
Low-ceiling diuretics	Bendroflumethiazide
Muscle relaxant	Baclofen
Opioid	Codein
	Pethidine
	Tramadol
Osmotically active laxatives	Lactulose
Other analgesic and antipyretic	Paracetamol
Other antineoplasic agents	Bortezomib
	Irinotecan
	Rituximab
Other cardiac preparations	Trimetazidine
Oral iron preparations	Ferrous sulphate
Other nervous system drug	Tetrabenazine
Potassium	Potassium chloride
Potassium-sparing agents, diuretics	Spironolactone
PPI	Omeprazole
	Rabeprazole
Psicoanaleptic antidepressant	Mirtazapine
Selective calcium channel blocker	Amlodipine
Synthetic anticholinergic	Glycopyrronium
	Mebeverine
Thyroid preparations	Levothyroxine
Vasodilator used in cardiac diseases	Glyceril trinitrate
	Isosorbide dinitrate
	Isosorbide mononitrate
Vitamin B1	Vitamin B1
Vitamin B12	Vitamin B12
Vitamin B-complex combinations	Vitamin B-complex
Vitamin D and analogues	Alfacalcidiol

Appendix 8

Standard procedure to culture *C. difficile* and test for antimicrobial susceptibilities on a clinical setting

Stool sample collection and storage according to hospital protocol for stool specimen collection and handling

- Stool must be freeze at -20◦C upon reception if it is not treated immediately (Standards Unit, 2014).
- If the sample was frozen, bring it to room temperature (25◦C) before continue to the next step (Standards Unit, 2014).

Culture procedure

- Perform alcohol shock method to obtain *C. difficile* spores alone as suggested by the Public Health England Standards for Microbiology investigation:
    - Prepare a 1: 1 suspension of stool sample and methylated spirit/absolute alcohol in a screw – capped glass bijou (De Silva, 2012).
    - Blend by vortexing and allow to rest at room temperature (25◦C) for 30 minutes.
    - With disposable pastette, inoculate 2 drops of the precipitate to the CCEY agar and streak for single colonies.
    - Repeat same procedure for the control organisms (ATCC strains).
    - Incubate the plates anaerobically at 36◦C±1◦C and read the plates for growth after 48 hours' incubation (Standard Unit, 2014).

Identification of colonies with API 20A

As specified by the manufacturer:

- Select a single colony with a sterile swab (The use of young cultures, 18-24 hours old, is recommended) and mix it on the API 20A ampule medium. The final turbidity should be greater than or equal to 3 McFarland.

- Use the suspension immediately after preparation.
- Prepare incubation box according to manufacturer instructions.
- Register the strain references on the elongated flap of the tray.
- With a sterile pipette, inoculate the strip with the suspension in the ampule of API 20 A medium following manufacturer directions.
- Place the lid on the tray and incubate for 24 hours at 36°C ± 2°C in an anaerobic jar.
- Add the requested reagents according to manufacturer.
- Read the 8-digit numerical profile and insert result in the database (V3.0) with the identification software.
- Proceed to the next step given in the system.
- Identify the code as *Clostridium difficile.*

Antibiotic sensitivity testing

As specified by manufacturer:

- Make a suspension of each isolate of *C. difficile* in Brucella broth to a McFarland turbidity standard number 1.
- Do the same for the reference strains with known Minimal Inhibitory Concentrations (MICs).
- Inoculate 5 Brucella agars for each isolate (supplemented with 5πg/ml haemin and 1 µg/ml Vitamine K1, pre-reduced for 18-24 hours anaerobically).
- Soak a swab into the inoculum suspension and remove excess fluid.
- Streak the entire agar surface three times, rotating the plate 60 degrees each time to eventually distribute the inoculum.

- Allow the surface to dry for approximately 15 minutes before applying the Etest®.
- Place 2 Etests® strips per plate are placed onto the surface opposite to each other of four plates and place one last strip (Clindamycin) onto the last plate. Incubate at 35-37 ◦C anaerobically for 18 -24 hours (except for clindamycin which needs 36 hours).
- After appropriate incubation read results where the edge of the inhibition ellipse intersects with the strip. Record the MIC as πg/ml and whether the isolate is susceptible, intermediate or resistant according to breakpoint values.
- Proceed likewise for reference strains.

Appendix 9

Results of the Chi-square test

			Outcome		
			Active	Carrier	Total
Gender	Male	Count	8	19	27
		Percentage	34.8%	45.2%	41.5%
	Female	Count	15	23	38
		Percentage	65.2%	54.8%	58.5%
Total		Count	23	42	65
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 0.669$, p = 0.413					

			Outcome		
			Active	Carrier	Total
Age	Less than 65 years	Count	9	18	27
		Percentage	39.1%	42.9%	41.5%
	More than 65 years	Count	14	24	38
		Percentage	60.9%	57.1%	58.5%
Total		Count	23	42	65
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 0.085$, p = 0.771					

			Outcome		
			Active	Carrier	Total
Onset	Community	Count	6	14	20
		Percentage	27.3%	33.3%	31.3%
	Health care	Count	16	28	44
		Percentage	72.7%	66.7%	68.8%
Total		Count	22	42	64
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 0.247$, p = 0.619					

			Outcome		
			Active	Carrier	Total
Use of probiotics	Yes	Count	3	4	7
		Percentage	13.0%	9.5%	10.8%
	No	Count	20	38	58
		Percentage	87.0%	90.5%	89.2%
Total		Count	23	42	65
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 0.192$, $p = 0.662$					

			Outcome		
			Active	Carrier	Total
Gastric acid suppression	Yes	Count	17	30	47
		Percentage	73.9%	71.4%	72.3%
	No	Count	6	12	18
		Percentage	26.1%	28.6%	27.7%
Total		Count	23	42	65
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 0.046$, $p = 0.831$					

			Outcome		
			Active	Carrier	Total
GI perturbation	Yes	Count	13	18	31
		Percentage	56.5%	42.9%	47.7%
	No	Count	10	24	34
		Percentage	43.5%	57.1%	52.3%
Total		Count	23	42	65
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 1.112$, $p = 0.292$					

			Outcome		
			Active	Carrier	Total
Immunosuppression	Yes	Count	9	16	25
		Percentage	39.1%	38.1%	38.5%
	No	Count	14	26	40
		Percentage	60.9%	61.9%	61.5%
Total		Count	23	42	65
		Percentage	100.0%	100.0%	100.0%
$X^2(1) = 0.007$, $p = 0.935$					