PREVALANCE AND MOLECULAR EPIDEMIOLGY OF *Clostridium difficile* INFECTION IN HOSPITAL UNIVERSITI SAINS MALAYSIA PATIENTS AND ELDERLY COMMUNITY SUBJECTS IN KELANTAN

DR NADIAH HANIM ZAINUL

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Dr Nadiah Hanim Zainul

MMed Internal Medicine

Department of Internal Medicine

School of Medical Sciences, Universiti Sains Malaysia

Health Campus, 16150 Kelantan, Malaysia

Introduction: An increase in the incidence of *Clostridium difficile* infection (CDI) in Western countries has come to prominence over the last 15 years. However awareness and surveillance of CDI in Asia remained poor with epidemiological data being scanty in Asia and in particular Malaysia. CDI is commonly associated with nosocomial infections, but community acquired CDI has been reported with increasing frequency lately. Despite the increase in incidence and severity of CDI, a recent survey found awareness of CDI being poor, with underestimation of its contribution to antibiotic-associated disease and recurrence rates.

Objective: The aims of this study were to explore the prevalence and associated risk factors of CDI in hospitalized patients in HUSM, to explore the carriage rate among the elderly in the community in Kelantan, and to determine the level of awareness of CDI among staff and students in HUSM.

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AAD	Antibiotics Associated Diarrhoea
C. difficile	*Clostridium difficile*
CCCNA	*C. difficile* Culture Cytotoxin Neutralization Assay
CDAD	*Clostridium difficile* Associated Diarrhoea
CDI	*Clostridium difficile* Infection
CDifAg	*Clostridium difficile* Antigen
CDifToxin	*Clostridium difficile* Toxin
CDT	Binary Toxin
CKD	Chronic Kidney Disease
COPD	Chronic Obstructive Pulmonary Disease
CRP	C-Reactive Protein
CPE	Cytopathic Effect
CT	Computed Tomography
DM	Diabetes Mellitus
DNA	Deoxyribonucleic Acid
EIA	Enzyme Immunoassay
FMT	Faecal microbiota transplantation
HIV	Human Immunodeficiency Virus
HUSM	Hospital Universiti Sains Malaysia
IBD	Inflammatory Bowel Disease
IDSA	Infectious Diseases Society of America
IgG	Immunoglobulin G
IHD	Ischaemic Heart Disease

LTCF	Long-Term Care Facilities
NAAT	Nucleic Acid Amplification Test
NG	Nasogastric
NHS	National Health Service
PCR	Polymerase Chain Reaction
PPI	Proton pump inhibitor
RT	Ribotype
TC	Toxigenic Culture
TcdA	Toxin A
TcdB	Toxin B
WBC	White Blood Cells

ABSTRACT

Introduction

An increase in the incidence of *Clostridium difficile* infection (CDI) in Western countries has come to prominence over the last 15 years. However awareness and surveillance of CDI in Asia remained poor with epidemiological data being scanty in Asia and in particular Malaysia. CDI is commonly associated with nosocomial infections, but community acquired CDI has been reported with increasing frequency lately. Despite the increase in incidence and severity of CDI, a recent survey found awareness of CDI being poor, with underestimation of its contribution to antibiotic-associated disease and recurrence rates.

Objective

We aimed to explore the prevalence and associated risk factors of CDI in hospitalized patients in HUSM. We also aimed to explore the carriage rate among the elderly in the community in Kelantan. Finally we aimed to determine the level of awareness of CDI among staff and students in HUSM.

Methodology

This study is divided into 3 arms. For the hospital arm, it was a prospective cross sectional study of CDI prevalence among 76 hospitalized patients in HUSM from 1st April 2015 until 30th September 2015.

For the community arm, it was a cross sectional study of *C. difficile* carrier prevalence among 138 elderlies from the community in Kelantan from July 2015 to September 2015.

For both arms, stools were tested for *C. difficile* antigen and toxin detection using *C. DIFF QUIK CHEK COMPLETE*®. The samples were then sent to Western Australia for culture and PCR for toxin genes and ribotyping for molecular epidemiology.

For the awareness survey, it was a cross sectional study of *C. difficile* awareness among 154 participants comprised of HUSM staff and students during an awareness campaign for *C. difficile* in HUSM on 6th August 2015. Data was obtained thru a self-administered questionnaire which was based from a previous international internet-based awareness study.

Result

For the hospital arm, 20 samples (26.3%) were positive for *C. difficile* antigen (CDifAg), 7 samples (9.2%) were positive for *C. difficile* toxin (CDifToxin) and 19 samples (25%) were positive from direct culture. Significant ribotype diversity with six distinct ribotype groups (QX001, UK 017, QX 002, QX 107, QX 117 and QX 463) were identified. Charlson Comorbidity Index, urea, creatinine, albumin and CRP level, duration of hospitalization, use of antibiotics, use of chemotherapy, underlying medical illness and fulminant severity were significantly associated with CDI using Simple Logistic Regression (P-value < 0.25). Further analysis with Multiple Logistic Regression showed significant association between CDI with age, duration of hospitalization and use of antibiotics (P-value < 0.05).

For the community arm, 2 samples (1.6%) were positive for both CDifAg and direct culture while negative for CDifToxin. Ribotying of the 2 samples showed unknown strain. From the study, it was found that the study population did not have high PPI and antibiotics use which were known CDI risk factors.

For awareness study arm, there was a low level of awareness on CDI with only 2.6% of 154 respondents able to correctly answer all questions correctly. Ironically a large proportion of the

participants (n = 73; 47.4%) considered *C. difficile* to be overestimated in their current practise. There was no significant association between level of awareness on CDI with age, gender and occupation i.e. being a clinician.

Conclusion

In conclusion, this study demonstrated that the prevalence rate for CDI in hospitalized patients in HUSM were 26.3% for CDifAg, 9.2% for CDifToxin and 25% for direct culture with 6 distinct ribotype strains; QX001, UK 017, QX 002, QX 107, QX 117 and QX 463 identified. Independent risk factors for CDI were age, duration of hospitalization and use of antibiotics. The carrier rate for *C. difficile* was 1.6% among the elderly in the community with unknown strain identified from PCR ribotyping. Low usage of PPI and antibiotics were seen in our study population and could explain the low prevalence rate of CDI in our study population.

Low awareness on CDI was seen among healthcare professionals in HUSM which was also seen internationally. Therefore CDI being an underdiagnosed and under recognised issue in the healthcare system is an issue that needs to be addressed by all parties.

ABSTRAK

Pengenalan

Peningkatan kes jangkitan *C. difficile* (CDI) di negara Barat dapat diperhatikan beberapa tahun kebelakangan ini. Walaupun CDI menunjukkan peningkatan yang ketara di seluruh dunia, data epidemiologi berkaitan CDI di Asia terutamanya Malaysia sangat mengecewakan. CDI biasanya dikaitkan sebagai jangkitan nosokomial, namun kes di dalam komuniti juga telah dilaporkan meningkat sejak kebelakangan ini. Kajian kesedaran di kalangan pengamal perubatan seluruh dunia menunjukkan kesedaran tentang CDI yang rendah.

Objektif

Untuk mengenalpasti kekerapan dan faktor risiko CDI di kalangan pesakit yang dimasukkan ke HUSM . Kajian ini juga bertujuan untuk mengkaji kekerapan pembawa *C. difficile* di kalangan warga emas di Kelantan. Kami juga bertujuan untuk menentukan tahap kesedaran mengenai CDI di kalangan staf dan pelajar di HUSM .

Methodologi

Kajian ini dibahagikan kepada 3 bahagian. Untuk kajian hospital , ia adalah satu kajian keratan rentas kelaziman di kalangan 76 pesakit yang dimasukkan ke hospital di HUSM dengan CDI dari 1 April 2015 dan akan berakhir pada 30 September 2015.

Untuk kajian masyarakat , ia adalah satu kajian keratan rentas kelaziman pembawa kuman *C. difficile* di kalangan 138 warga emas di dalam masyarakat di Kelantan dari bulan Julai 2015 hingga September 2015.

Sampel najis telah diuji untuk *C. difficile* antigen dan toxin menggunakan *C. DIFF QUIK CHEK COMPLETE*®. Sampel najis kemudiannya telah dihantar ke makmal di Western Australia dan seterusnya dikultur dan menjalani ujian ribotaip bagi epidemiolgi molekular.

Bagi kajian kaji selidik kesedaran CDI, ia merupakan satu kajian keratan rentas di kalangan 154 peserta yang terdiri daripada kakitangan HUSM dan pelajar yang menghadiri kempen kesedaran *C. difficile* di HUSM pada 6 Ogos 2015. Data telah diperoleh menerusi borang soal selidik yang berdasarkan daripada kajian kesedaran peringkat antarabangsa yang telah berlangsung sebelumnya.

Keputusan

Untuk kajian hospital, 20 sampel (26.3%) didapati positif *C. difficile* antigen, 7 sampel (9.2%) positif untuk *C. difficile* toksin dan 19 sampel (25%) positif daripada kultur secara terus. Enam kumpulan ribotype berbeza (QX001 , UK 017, QX 002, QX 107, 117 dan QX QX 463) telah dikenal pasti. Indeks 'Comorbidity Charlson', urea, kreatinine, albumin dan CRP, tempoh rawatan di hospital, penggunaan antibiotik, penggunaan kemoterapi, pesakit yang mengalami penyakit perubatan kronik dan tahap penyakit fulminan mempunyai hubungan yang signifikan dengan CDI menggunakan Simple Logistik Regresion (Nilai-P < 0.25). Analisis lanjut dengan Multiple Logistic Regression menunjukkan hubungan yang signifikan antara CDI dengan usia, tempoh rawatan di hospital dan penggunaan antibiotik (Nilai-P < 0.05).

Untuk kajian masyarakat, 2 sampel (1.6%) adalah positif bagi CdifAg dan kultur secara terus manakala negatif untuk CDifToxin. Ribotying daripada 2 sampel tersebut menunjukkan ia tergolong didalam strain yang tidak dikenalpasti. Dari kajian ini, didapati bahawa populasi kajian mempunyai penggunaan antibiotik serta PPI yang rendah.

Untuk kajian kaji selidik kesedaran CDI, terdapat tahap kesedaran yang rendah mengenai CDI dengan hanya 2.6% daripada 154 responden dapat menjawab semua soalan dengan betul. Sebahagian besar daripada peserta (n = 73; 47.4%) menganggap *C. difficile* tidak penting di dalam amalan semasa mereka. Tidak ada hubungan yang signifikan antara tahap kesedaran mengenai CDI dengan usia, jantina dan pekerjaan.

Kesimpulan

Pada kesimpulannya, kajian ini menunjukkan bahawa kadar kelaziman untuk CDI adalah 26.3% untuk CDifAg, 9.2% untuk CDifToxin dan 25% untuk kultur secara terus untuk pesakit di dalam wad. Enam jenis ribotaip yang berbeza iaitu QX 001 , UK 017, QX 002, QX 107, QX 117 dan QX 463 telah dikenal pasti. Faktor-faktor risiko yang mempunyai kaitan dengan CDI adalah umur, tempoh rawatan di hospital dan penggunaan antibiotik.

Kadar kelaziman pembawa *C. difficile* didapati sebanyak 1.6% di kalangan warga tua di dalam masyarakat. Ribotype dari PCR menunjukkan ia tergolong didalam strain yang tidak dapat dikenal pasti. Penggunaan PPI dan antibiotik yang rendah dapat dilihat di dalam populasi kajian ini dan dapat menjelaskan kadar kelaziman yang rendah di dalam populasi kajian ini.

Tahap kesedaran yang rendah mengenai *C. difficile* di kalangan warga HUSM juga dapat dilihat di peringkat antarabangsa. Oleh itu kepentingan mencegah CDI kurang diberi tumpuan dan pengiktirafan di dalam sistem penjagaan kesihatan dan ini merupakan satu isu yang perlu diberi perhatian oleh semua pihak.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 *Clostridium difficile* (*C. difficile)* – The organism

C. difficile is an opportunistic, gram-positive, rod-shaped, spore-forming anaerobic bacterium that exists in the soil and the gastrointestinal tract of animal and humans. It is part of the normal intestinal microbiota in 1–3% of healthy adults and 15–20% of infants (Goudarzi et al., 2014). It was first discovered as a new species of bacteria in 1935 by Hall and O'Toole and was named *Bacillus difficilis* due to its difficult anaerobic isolation from human faeces. At that time it was still not identified as a causative agent of human disease and was noted as a component of normal faecal microbiota of newborn infants (Hall and O'Toole, 1935). Forty years later in the 1970s, it was then discovered that the microorganism was able to produce toxins causing toxin-mediated infection thus its name was subsequently changed to *Clostridium difficile* (Kuipers and Surawicz, 2008).

The bacteria can exist in spore form where it can survive harsh environments and common sterilization techniques making it resistant to high temperatures, ultraviolet light, harsh chemicals and antibiotics. Pathogenic strain of *C. difficile* produces cytotoxin (toxin A and toxin B) which results in varieties of pathology from being asymptomatic to mild or moderate diarrhoea, to fulminant and sometimes fatal pseudomembranous colitis.

1.2 Epidemiology of *Clostridium difficile* infection (CDI)

1.2.1 Nosocomial *Clostridium difficile* infection

C. difficile is one of the most common nosocomial infections in recent decades, with increasing prevalence, morbidity and mortality being reported worldwide (Kachrimanidou and Malisiovas, 2011). Colonisation occurs in 20-40% of hospitalised adults compared with 2-3% in healthy adults (Heinlen and Ballard, 2010). Studies have also found high rates of

asymptomatic Colonisation in neonates and elderly with prevalence ranging from 40-84% (Karasawa et al., 2005, Hensgens et al., 2012b).

The incidence of CDI has progressively increased. Prevalence of CDI has been reported at 8.75 cases/1,000 adult admissions in United States hospitals. (Chung et al., 2010). Data from the Canadian nosocomial surveillance between November 2004 and April 2005 showed incidence of 65 cases per 100,000 patient-days, or 4.6 cases per 1,000 admissions with a large outbreak in 2003 showing a 4-fold increase in the incidence of CDI (22.2 cases per 100,000 population in 1991 to 92.2 cases per 100,000 in 2003) (Gilca et al., 2010).

Since 2001, outbreaks in North America have been attributed to a hypervirulent strain of *C. difficile*, referred to as ribotype BI/NAP1/027 (toxinotype III). Ribotype 027 has since spread beyond North America to Europe and Australia, and has been reported in several more developed Asian countries (Clements et al., 2010, Rupnik et al., 2009). The increased incidence of infections is accompanied by greater severity of disease caused by this strain, with higher case-fatality rates and related morbidity (Clements et al., 2010).

Unfortunately data on the incidence of *C. difficile* infection in Asia in particular are limited but the available reports suggest an increasing number in countries including Singapore, Taiwan, Korea, India and Japan. Limited studies indicate that CDI may also be a significant pathogen in this region, but the true prevalence of CDI remains unknown particularly in South-East Asia. In the Asia Pacific region, CDI prevalence had increased over the years. In Singapore, the prevalence increased from 1.49 cases per 10,000 patient-days in year 2001 to 6.64 cases per 10,000 patient-days in 2006 (Lim et al., 2008), while in Malaysia, a study observed the incidence of CDI cases in northern eastern coast of Malaysia at 13.7% (Hassan et al., 2012a). A study in Thailand in 2012 among 175 patients showed that 26.9% were positive for antigen while 12.6% were positive for toxins (Putsathit et al., 2015).

1.2.2 Community acquired *Clostridium difficile* infection

C. difficile has traditionally been linked to disease in hospitalised populations. However community acquired CDI also occurs and recently has been reported with increasing frequency (Hensgens et al., 2012b). An estimated 20 % to 28 % of CDI is community associated with an incidence of 20 to 50 cases per 100 000 population in the United States, Sweden and England (Kutty et al., 2010, Karlström et al., 1998, Wilcox et al., 2008). Previous studies have shown that approximately 40% of patients acquiring community-associated CDI were not exposed to traditional risk factors for CDI such as advanced age, antibiotic exposure, and medications to suppress gastric acid suggesting that additional factors may contribute to infection. Possible reservoirs for community-associated disease include soil, water, pets, meat and vegetables (Heinlen and Ballard, 2010). Transmission in food has been proposed as an explanation for community-acquired disease with *C. difficile* being isolated from food. The predominant strain isolated in food are ribotypes 027 and 078, strains well established to cause human disease (Carroll, 2011). Community-acquired infections are more commonly associated with binary toxin-producing strains such as 078 (Riley, 2006).

1.2.3 Epidemiology in Malaysia

Systematic search of PubMed currently yields only four papers mentioning isolation of *C. difficile* in Malaysia, three of which were published prior to 1997 (Boey et al., 1997, Parasakhti et al., 1988, Hassan and Cheng, 1991). The most recent publications introduced systematic testing for CDI in a hospital in North Eastern Malaysia (Hassan et al., 2012a, Hassan et al., 2012b). The prevalence of *C. difficile* was 13.7% among 175 stool samples tested by assay for toxin A and B. No data on carriage of *C. difficile* among elderly in the community are available in Malaysia but this data may be important to explain the high prevalence of *C. difficile* infection in hospitals.

1.2.4 PCR ribotypes in Asia

Ribotyping data with internationally recognised nomenclature are available for China, Japan, Singapore, Hong Kong, Taiwan and Korea. Overall, the most prevalent ribotypes in Asia appear to be 017, 018, 014, 002, and 001 (Collins et al., 2013). These ribotypes are among the top ten most commonly found ribotypes in Europe.

RT 027, which is still the major ribotype in North America, has been reported only sporadically in Hong Kong, Japan, South Korea, Singapore and more recently, China. Similarly, RT 078 has only been reported in South Korea and China. RT 017, which is a toxin A-negative, toxin B-positive (A−B+) strain, is the predominant strain in China and South Korea and is prevalent in Japan, Taiwan and Hong Kong. This ribotype has also caused epidemics in The Netherlands and Ireland and is an emerging ribotype in Australia (Collins et al., 2013).

1.3 Pathogenesis of *Clostridium difficile* infection

C. difficile spores are transmitted from person to person via the faecal-oral route. Bacterial spores are metabolically dormant and are resistant to desiccation, chemicals and extreme temperatures. Spores frequently contaminate the environment around patients with CDI, potentially persisting for months and even years.

There are two forms of the organism, a dormant spore form that is resistant to antibiotics and a vegetative form that can produce toxins and is susceptible to the activity of antibiotics. The vegetative form of *C. difficile* is killed at normal gastric pH (defined as a pH < 4.0), whereas *C. difficile* spores may survive exposure to acid in the stomach (McFarland et al., 2007). Spores that do pass through the stomach germinate to their vegetative form in the small intestine. Indigenous colonic flora is the first line of defence against Colonisation by pathogens such as *C. difficile*. This can be disrupted by antimicrobial drugs, several medications (i.e., chemotherapy drugs, proton pump inhibitors), illness or surgical procedures. This disruption

allows *C. difficile* to colonize the intestinal tract, reproduce and cause clinical disease (Sunenshine and McDonald, 2006). However, only toxigenic strains are associated with the development of *C. difficile* diarrhoea as some *C. difficile* strains do not produce toxins and therefore do not cause disease.

In summary the pathogenesis of CDI consist of alteration of the normal faecal flora, Colonisation with toxigenic *C. difficile* and growth of the organism with elaboration of its toxins.

1.3.1 Factors affecting virulence

The virulence of the infecting strain and the host's immune response determine whether a person develops clinical disease and also determines the severity of disease. *C. difficile* virulence factors include toxin production, sporulation, surface layer proteins and adherence, and toxin variant strain.

1.3.1.1 Toxins

The primary virulence factor of *C. difficile* is its ability to produce and release two toxins namely Toxin A (TcdA) and Toxin B (TcdB). Both toxins are cytotoxic and stimulate production of tumour necrosis factor and pro-inflammatory interleukins which result in inflammation and increased vascular permeability in the colon, the release and accumulation of neutrophils and pseudo-membrane formation (Poutanen and Simor, 2004). TcdA play a more critical role in the pathogenesis of *C. difficile* diarrhoeal disease than TcdB. TcdA has been shown to be more closely associated with tissue damage and fluid accumulation (Bongaerts and Lyerly, 1994, Johnson et al., 1990). These models also suggest that TcdB may contribute to disease only after TcdA has damaged the gastrointestinal wall. However, they also observed that either one of the two toxins alone can cause disease (Poxton et al., 2001,

Bongaerts and Lyerly, 1994, Barbut et al., 2002). It was evidenced by outbreaks of severe infection caused by TcdA negative; TcdB positive strains.

Approximately 6 to 12.5% of strains of *C. difficile* produce another toxin, called *C. difficile* transferase (CDT) or binary toxin that is unrelated to either TcdA or TcdB. CDT alone does not appear to cause disease. The clinical significance of CDT in CDI remains uncertain (Karen C and John G, 2011). More recent findings however indicate that CDT contributes to an increased severity of disease as CDT production appears to be epidemiologically associated with strains producing higher fatality rates (McDonald et al., 2005, Shen, 2012, Dingle et al., 2011).

1.3.1.2 Sporulation and germination

The rate of *C. difficile* sporulation is an important virulence factor. 'Hyper-sporulation', in addition to the ability of spores to survive in the environment, propagates the spread of *C. difficile* from person-to-person. Therefore it has been postulated that increased sporulation may be associated with hypervirulence (Merrigan et al., 2010, Dawson et al., 2011).

1.3.1.3 Surface layer proteins and adherence

Surface proteins are integral to the adherence of the organism to the gut mucosa and can induce both inflammatory and antibody responses in the host (Drudy et al., 2004, Calabi and Fairweather, 2002, Ausiello et al., 2006). There is considerable variability between the surface proteins of different strains. Therefore the differences in these proteins may alter a particular strain's ability to adhere to intestinal epithelial cells (Calabi and Fairweather, 2002, Drudy et al., 2004, Péchiné et al., 2005).

1.3.1.4 Toxin variant strains, Ribotype 027 , Ribotype 078

C. difficile can be divided into 5 genetic groups or clades (designated clades 1 to 5) that continue to evolve at the strain level giving rise to hypervirulent types. Examples of these strains are ribotype 027 (toxinotype III, ST-1, BI/NAP1) and ribotype 078 (ST-11) strains which are associated with more severe disease and higher mortality rate (He et al., 2013).

Since early 2000, outbreaks in healthcare facilities have spread across the United States, Canada and Europe attributable to this new highly virulent strain aka *C. difficile* ribotype 027. Mortality rates in outbreaks caused by this strain have been three-times higher than in outbreaks caused by less virulent strains (Pépin et al., 2005, Loo et al., 2005, Warny et al., 2005). This strain has unique characteristics that may explain the virulence with higher levels of toxin production, fluoroquinolone resistance and the production of binary toxin. It produces a binary toxin and has a partial deletion in a toxin regulator gene i.e. TcdC that cause hyperproduction of TcdA and TcdB *in vitro* (Warny et al., 2005, Åkerlund et al., 2008). Aside from having altered TcdC, epidemic 027 strains have five unique genetic regions not present in historical 027 strains (Stabler et al., 2006). These genes include mutations that explain enhanced toxicity, motility, survival and increased sporulation. Epidemic isolates of *C. difficile* ribotype 027 were resistant to fluoroquinolones (McDonald et al., 2005, Loo et al., 2005), which suggests that the increased use of quinolones may have influenced the emergence of this strain.

Ribotype 078 (toxinotype V) has also contributed to the increased incidence of CDI over the past 10 years. It is the most common strain isolated from pigs and cattle in the USA (Keel et al., 2007) and is now the third most common ribotype causing disease in humans in Europe (Freeman et al., 2010). These strains also produce CDT and carry a 39-bp deletion in TcdC in combination with a point mutation at position 184 resulting in a stop codon (Freeman et al., 2010).

Ribotypes 027 and 078 produce TcdA and TcdB in higher quantities than other strains, probably due to the mutated TcdC protein, which has been shown to be non-functional in ribotype 078 strains (Carter et al., 2011).

1.3.1.5 Host immune response

Host immune response influences the clinical expression of *C. difficile* infection. Human immune response to *C. difficile* develops during infancy. Infants who carry *C. difficile* develop antibodies to TcdA and to TcdB. In adults, high titres of serum immunoglobulin G (IgG) against TcdA promote the development of an asymptomatic carrier state rather than infection (Kyne et al., 2000). When infection develops, person with high antibody concentrations tend to have shorter durations of illness and less risk of recurrence than person who lack these antibodies. Individuals without prompt development of these antibodies to TcdA are more likely to experience more severe symptoms and have an increased risk for recurrence of CDI (Kyne et al., 2001, Katchar et al., 2007).

1.4 Risk factors of *C. difficile* infection

Risk factors for development of CDI are advanced age of 65 years old and more, duration of hospitalization, exposure to antimicrobial agents, use of acid suppressing medications, chemotherapy, underlying comorbidity and gastrointestinal surgery or manipulation of the gastrointestinal tract (including tube feeding) (Cohen et al. 2010).

1.4.1 Drug related risk factors

Drugs that have been implicated in *C. difficile* infections are antibiotics, immunosuppressive agents, proton pump inhibitors and cancer therapeutics.

1.4.1.1 Antibiotics use

Exposure to antibiotics is the preeminent risk factor. More than 90% of healthcare associated *C. difficile* infections are associated with antibiotic use (Sunenshine and McDonald, 2006). Administration of broad-spectrum antimicrobials causes disruption to the normal intestinal flora and subsequently promote proliferation of toxigenic *C. difficile.* Historically, clindamycin was the first antibiotic implicated in CDAD when it was associated with pseudomembranous colitis in the early 1970s (Pear et al., 1994). Now it is well known that penicillin, ampicillin, cephalosporin and fluoroquinolones can all precipitate CDAD. Studies carried out after 1980 showed that cephalosporin is the most common agent implicated in nosocomial CDAD. A recent meta-analysis by Riley et al. indicate that third-generation cephalosporin remain the strongest antibiotic risk factor (Slimings and Riley, 2014). In outpatient settings, antibiotics such as ampicillin, amoxicillin or amoxicillin-clavulanate combination are important and common causes. Less commonly implicated antibiotics are macrolides, tetracyclines, sulphonamides, trimethoprim, chloramphenicol and penicillin other than ampicillin/amoxicillin (Vaishnavi, 2009).

Risk of development of CDI is also increased with multiple antibiotics use and longer course of therapy. The number of administered antibiotics, their dosage and the duration of therapy have been identified as factors determining the risk for CDI (Owens et al., 2008). CDI risk is elevated 7 to 10 fold during antibiotic therapy and the first month after cessation of antibiotics. It remains elevated for at least 3 months after administration of antibiotics (Hensgens et al., 2012a).

1.4.1.2 Proton pump inhibitors

Colonisation of normally sterile upper gastrointestinal tract can be a consequence of gastric acid suppressive use. Lower acidity environment allows vegetative forms of *C. difficile* to

survive. Patients are about twice as likely to develop CDAD with PPI, due to increased survival of spores. Recent meta-analyses confirm the association of PPI use with an increased risk of CDI. A meta-analysis by Janarthanan showed a 65% increase in the incidence of CDAD among PPI users while another meta-analysis by Kwok et al showed an increase of 85% (Janarthanan et al., 2012, Kwok et al., 2012).

1.4.1.3 Cancer chemotherapy

Patients undergoing antineoplastic chemotherapy are at increased risk for CDI. Administration of cancer chemotherapeutic agents possessing antibacterial properties may also result in sufficient disturbance of the intestinal micro flora to allow Colonisation with *C. difficile*. Some research have suggested that this association may be related to concurrent use of antimicrobials and immunosuppression rather than to the use of chemotherapeutic drugs alone (Toor et al., 2001, Arango et al., 2006).

1.4.1.4 Immunosuppressive agents

Immunosuppressive drugs have been reported to be associated with the development of CDAD. Patients receiving immunosuppressive drugs are debilitated and therefore are unable to mount an effective IgG antibody response against *C. difficile t*oxin thereby increasing the risk for CDAD. Though the ability to mount an immune response is not protective against *C. difficile* Colonisation, it is associated with decreased morbidity, mortality and recurrence of CDAD (Kyne et al., 2001). Patients at highest risk for fulminant disease among others include those who have recently received immunosuppressive therapy besides have undergone surgical procedures or those with a history of CDAD (Bartlett, 2002, Dallal et al., 2002).

Exposure to corticosteroids is significantly associated with an increased risk of CDAD relapse warranting a longer treatment course. *C. difficile* Colonisation is more frequent in intensive

care and oncology units where use of broad spectrum antibiotic and immunosuppression are wide spread. The use of immunosuppressive may account for a large number of CDAD patients without prior use of antibiotics. As the use of immunosuppressive increases, the incidence of CDAD will also rise further (Vaishnavi, 2009).

1.4.2 Non-drug related risk factors

Non-drug related risk factors can be further divided into host risk factors, environmentally related risk factors and pathogen related risk factors. Host risk factors include age, underlying co-morbidity, impaired immunity and prolonged hospital stay.

1.4.2.1 Age

The elderly population is at higher risk of developing CDI. Advanced age is one of the most commonly-cited risk factors for CDI. A study reported that patients over 65 years of age had a 10-fold higher risk for CDI during an outbreak than did younger patients (Pépin et al., 2005). There is also a trend of increasing CDI related hospital discharges among persons greater than 65 years of age compared to those of other age group (McDonald et al., 2006). Collectively, higher incidence and severity of infection among older persons are most likely related to the increased likelihood for older persons to have a greater number of comorbid conditions, more severe illness, suppressed immune systems and hospitalization or residence in long-term-care settings as compared with younger persons.

1.4.2.2 Prolonged Hospital Stay

The spread of *C. difficile* within hospitals is well-documented. Early studies showed that hospitalised populations exhibit much higher rates of Colonisation, with one study reporting that hospitalised adults have a 20-40% rate of Colonisation compared with a rate of 2-3%

among healthy adults (McFarland et al., 1989, Viscidi et al., 1983). The risk of *C. difficile* Colonisation increased proportionately with length of hospital stay. It is estimated to be 13% in patients with hospital stays of up to two weeks and 50% in those with hospital stays longer than four weeks (Clabots et al., 1992). Patients who share a room with a *C. difficile*–positive patient acquire the organism after an estimated hospital stay of 3.2 days, compared with a hospital stay of 18.9 days for other patients (McFarland et al., 1989).

1.4.2.3 Co-morbidity

A number of specific comorbid conditions are associated with CDI. Specific comorbid conditions that have been associated with CDI include gastrointestinal diseases, COPD, malignancy, renal disease or failure, diabetes, HIV and conditions resulting in an immunocompromised state (Morris et al., 2002, Dial et al., 2008). Agency for Healthcare Research and Quality (AHRQ) data showed that multiple co-morbidities put patients at risk for CDI (Elixhauser and Jhung, 2008).

A study within a hospital with endemic CDI found that myocardial infarction, COPD, liver disease, renal failure, and leukaemia and/or lymphoma were associated with increased risk for CDI (Dubberke et al., 2007b). However, it is unclear whether the increased risk for CDI is due to the actual condition or due to treatment for sequela of their chronic illness such as antimicrobials use thus increasing risk for CDI among these persons (Cunney et al., 1998, Pituch, 2009). Co-morbidity that contribute to the risk of dying within 30 days include cognitive impairment and liver, renal and ischemic heart diseases (Welfare et al., 2011).

Persons with chronic underlying conditions are also likely to seek medical care in healthcare facilities more often than persons who do not have comorbid conditions. These exposures to healthcare facilities may increase the likelihood for them to be exposed to surfaces and persons

contaminated with *C. difficile*, thus increasing their risk for CDI for a reason other than their specific underlying illness.

Persons with gastrointestinal conditions are considered to be at particular risk for CDI. Identifiable risk factors involving gastrointestinal diseases are IBD, bowel ischaemia, mechanical bowel cleansing, enteric infections that change colonic microflora, prolonged presence of a nasogastric tube for enteral feeding, use of electronic rectal thermometers, use of enemas, gastrointestinal stimulants and stool softeners. Studies have found increasing incidence rates among patients with IBD exceed those in the general hospitalised population (Rodemann et al., 2007). *C. difficile* is estimated to be the cause of 5-19% of IBD flares. Between 1998 and 2004, the highest prevalence rate of CDI occurred among patients with ulcerative colitis (37.3 per 1,000) followed by patients with Crohn's disease, patients with non-IBD gastrointestinal conditions, and finally, the general medical population within the National Health Service (NHS) (Meyer et al., 2004). The mortality rate was four times higher among hospitalised patients who had IBD and CDI than among patients hospitalised for IBD alone and was two times higher among patients hospitalised with CDI alone (Koss et al., 2006, Ananthakrishnan et al., 2008).

Another established gastrointestinal related risk factor for CDI is manipulation of the gastrointestinal tract which includes tube feeding. Bliss et al. studied the incidence of *C. difficile* acquisition and CDAD in tube-fed and non-tube fed patients and reported that tube-fed patients, especially those receiving post pyloric tube feeding are at greater risk for development of CDAD compared with hospitalised, non-tube-fed patients (Bliss et al., 1998).

Renal impairment is also one of the risk factor for CDI. Declining renal function is associated with impairments in adaptive and innate immunity with resultant increased susceptibility to infections and infection-related morbidity and mortality. Uraemia affects cell-mediated as well as innate and adaptive immunity Therefore they are at increased risk of infection and more

likely to receive antibiotic therapy. Additionally, individuals with renal insufficiency have reduced gastric acid secretion which may increase the risk of Colonisation with *C. difficile* (Mullane et al., 2013).

1.4.2.4 Charlson Comorbidity Index

Comorbidities, in general, are medical conditions that underlie the primary illness for which a person is seeking medical attention. These medical conditions increase a person's total burden of disease, are likely to contribute to risk of complications or death, and may affect physician choice of treatment for other illness (Foley et al., 1992). The collective effect of multiple comorbid conditions was assessed through the use of the Charlson Comorbidity Index. The Charlson Comorbidity Index was first developed as a weighted index which was shown to predict one-year mortality in a small cohort of hospitalised patients (Charlson et al., 1987). The index assigns a weight to each of the 19 conditions based on their potential for increasing the likelihood of death. Each patient's specific conditions are identified, at which point the weights for comorbidities are added to serve as a summary score. This summary score takes into account both the number of conditions and the risk associated with these conditions into account. A higher score represents higher levels of comorbidity (Charlson et al., Mackenzie, 1987).

1.5 Clinical presentation of *Clostridium difficile* infection

CDI manifest with a spectrum of clinical conditions which range from asymptomatic carriage, mild or moderate diarrhoea, to fulminant and sometimes fatal pseudomembranous colitis, toxic megacolon and death (Cohen et al., 2010). Patients with clinical symptoms can be stratified into mild to moderate illness, severe illness and fulminant disease (Bartlett and Gerding, 2008, Cohen et al., 2010).

1.5.1 Asymptomatic carriage

Colonisation with *C. difficile* is the presence of the organism in a person with no clinical symptoms e.g. diarrhoea. Symptomatic disease is less often seen in carriers, despite the fact that most of the *C. difficile* isolates are toxin producing (Shim et al., 1998). They however may be a reservoir of *C. difficile* and contribute to disease transmission especially in long-term care facilities (Riggs et al., 2007). Based on several studies, the frequency of carrier stage in healthy adults, hospitalised patients, and patients with long hospital stays are approximately 1-3%, 20-30%, and 50% respectively (Nakamura et al., 1981, Karasawa et al., 2005, Riggs et al., 2007).

1.5.2 Mild to moderate CDI

Mild disease is characterized by diarrhoea in the absence of signs and symptoms of colitis. Patients with moderate disease have diarrhoea with evidence of colitis characterized by fever and abdominal cramps, usually in the lower quadrants. Laboratory abnormalities in mild and moderate disease include a leucocytosis level of 15,000 cells/μl or lower and a serum creatinine level less than 1.5 times the premorbid level (Cohen et al., 2010, Bartlett and Gerding, 2008). For mild-to-moderate disease, diarrhoea is usually the only symptom. The incubation period from ingestion of *C. difficile* to onset of symptoms has been estimated to be a median of 2–3 days (McFarland et al., 1989). Patients may experience multiple episodes of diarrhoea but usually considerably less than 10 per day. Watery stool with a characteristic foul odour is the usual presentation although mucoid or soft stools may also occur. Presence of gross blood in the stool is rare (Bartlett, 2002). Systemic symptoms are usually absent in mild disease and physical examination is remarkable only for mild abdominal tenderness (Sunenshine and McDonald, 2006). Other clinical features consistent with CDI include abdominal cramps, fever, leucocytosis, and hypoalbuminemia. Fever occurs in 28%, leucocytosis in 50% and abdominal pain in 22% of cases (Bartlett et al., 1980).

1.5.3 Severe CDI

Infectious Diseases Society of America (IDSA) 2010 guidelines criteria for severe CDI also took into account blood investigation as part of the criteria on the basis of WBC greater than 15 x 10^9/L, or a level of creatinine 1.5-fold above the patient's baseline value (Cohen et al., 2010). Other characteristics of severe disease include markedly elevated temperature reaching 40°C, pseudomembranous colitis and hypoalbuminemia (serum albumin level of <25 g/L).

Around 10% of cases of CDI have clinical features consistent with severe CDI (Muto et al., 2005). Severe disease usually present with profuse, non-bloody diarrhoea, abdominal pain, fever, nausea, anorexia, malaise and abdominal tenderness. It may also cause paralytic ileus that can evolve into toxic megacolon (Sunenshine and McDonald, 2006).

In up to 20% of patients with severe CDI, diarrhoea and fluid loss are minimal and patients present instead with abdominal distention and ileus which is in contrast to mild form of CDI, therefore often leading to misdiagnosis (Dallal et al., 2002). This occurs when the infection causes paralytic ileus, preventing the passage of stool and most common in postoperative patients who are receiving narcotics for pain (Bartlett and Gerding, 2008). Therefore even in the absence of diarrhoea, symptoms such as unexplained fever, leucocytosis and abdominal pain in a patient with recent antibiotic exposure should raise suspicion of CDI.

A study in Boston showed that patients with severe *C. difficile* colitis were more likely to have abdominal pain, tenderness and distention, peritonitis, hemoconcentration, hypoalbuminemia and an elevated (> 25 x 10^9/L) or suppressed (< 1.5 x 10^9/L) white blood cell count (Rubin et al., 1995). Another study showed C-reactive protein and leukocytes can be moderately or even highly elevated in 58 % of patients with unexplained leucocytosis but had CDI (Wanahita et al., 2003). Hypoalbuminemia is also a common feature as CDI is a protein-losing enteropathy and low albumin is considered a marker of inflammatory states.

1.5.4 Fulminant CDI

Fulminant colitis occurs in approximately 3% of CDI patients and is associated with severe complications which include perforation, peritonitis, prolonged ileus, megacolon and death. It presents with signs and symptoms of severe toxicity i.e. fever, colitis with severe lower quadrant or even diffuse abdominal pain, diarrhoea, distension and marked leucocytosis (Triadafilopoulos and Hallstone, 1991, Kelly et al., 1994). The timing from onset of any CDI symptoms to fulminate colitis varies from weeks to just a couple of hours of which patients with rapid progression have worse outcomes (Dallal et al., 2002). CDI is associated with exacerbation of ulcerative colitis and it was observed that fulminant colitis was reported more frequently during outbreaks of *C. difficile* in patients with IBD which carries higher mortality than those without underlying IBD (Hookman and Barkin, 2009).

1.6 Diagnosis of *Clostridium difficile* infection

Diagnosis of CDI is based on both clinical and microbiological methods with the presence of diarrhoea and stool test positive for toxigenic *C. difficile* or its toxin, colonoscopy, or histopathologic findings demonstrating pseudomembranous colitis (Cohen et al. 2010). A recent guideline by the SHEA/IDSA recommended all the laboratory tests be done on the unformed stool specimens unless ileus is suspected (Cohen et al., 2010). Only watery or loose stools should be tested for *C. difficile* because the rate of asymptomatic Colonisation is relatively high; therefore, testing in persons who do not have diarrhoeal symptoms may identify patients who are colonized but not infected.

1.6.1 Laboratory diagnosis

Diagnosis of CDI is a clinical diagnosis supported by laboratory findings. Routine laboratory tests for CDI diagnosis include a cytotoxin assay for Toxin B, a rapid enzyme immunoassay

(EIA), a latex agglutination test to detect bacterial antigen and anaerobic stool culture. For the past 30 years, the two primary reference tests are the *C. difficile* culture cytotoxin neutralization assay (CCCNA) and toxigenic culture (TC) (Planche and Wilcox, 2010, Sambol et al., 2000). CCCNA detects the presence of *C. difficile* toxins, toxin B and toxin A, in cell culture. If toxin-induced cytopathic effect (CPE) is observed, *C. difficile* is confirmed as the cause of infection by performing a neutralization assay to ensure that the CPE is attributable to *C. difficile* toxins rather than nonspecific toxicity by using either *C. sordellii* or *C. difficile* antiserum. The reported sensitivities for CCCNAs range from 65 to 90% (Burnham and Carroll, 2013). However the downside of the test is the relatively long turnaround time due to the technical demands of the lab procedures (O'Connor et al., 2001).

Toxigenic culture (TC) is based upon isolating the organism from faecal specimens and determining if the recovered isolate is a toxin-producing strain (Burnham and Carroll, 2013). *C. difficile* culture alone is not sufficient because not all *C. difficile* strains produce toxin (Rea et al., 2012, Viscidi et al., 1983, Burnham and Carroll, 2013). In studies that have evaluated the use of TC performed after a negative direct toxin test, an increased yield of *C. difficile* by 15-23% has been seen (Carroll, 2011). TC has several utilities. It is important for organism characterization in the setting of an outbreak or other epidemiological studies; when evaluating a new test method; for surveillance of drug resistance and to evaluate new therapies; and occasionally for difficult patient management. The SHEA/IDSA guidelines support the use of toxigenic culture as the gold standard in method comparison studies (Cohen et al., 2010) but the labour requirement and turnaround time are not usually considered practical for routine diagnostic use (Burnham and Carroll, 2013).

The enzyme immunoassay (EIA) for detection of toxins A and B has been the most widely used diagnostic test for CDI because of its rapid turnaround, low cost and simplicity. There are a number of commercially available EIAs for *C. difficile* toxin. However the sensitivity and

specificity that have been reported for these assays vary widely, from approximately 40% to 100% (Burnham and Carroll, 2013). Compared with TC, EIAs for toxins A and B have low sensitivity from 60% to 80% and a specificity from 91% to 99.4% (Eastwood et al., 2009) while in comparison with CCCNA, sensitivity of 75% to 95% and a specificity of 83% to 98% have been reported (Goudarzi et al., 2014). Different strains of *C. difficile* can also provide different results in toxin EIA assays (Tenover et al., 2010). Due to the inadequate sensitivity of toxin EIAs for *C. difficile* testing, it is not considered the best way to make a diagnosis of CDI (Eastwood et al., 2009, Cohen et al., 2010, Carroll, 2011). The SHEA/IDSA guideline outlines that EIAs should no longer be considered adequate stand-alone tests for the diagnosis of *C. difficile* infection (Cohen et al., 2010).

C. difficile nucleic acid amplification test (NAATs) is the most recent method for detection of *C. difficile*. It identifies genes (not the toxin) that encode the toxins (usually toxin B) by using PCR or loop-mediated isothermal amplification of DNA. These assays have a short turnaround time, and sensitivities range from 84% to 96% and specificities range from 94 to 99%. (Deshpande et al., 2011).

1.6.2 Endoscopy and radiology

C. difficile most often causes a nonspecific colitis. In more severe cases, the distinct macroscopic appearance of pseudomembranous colitis at endoscopy or by histopathologic examination may be seen. At least 90 % of patients with pseudomembranous colitis demonstrate either *C. difficile* or its toxins in stool samples (Wolfhagen et al., 1994). Milder cases may only reveal nonspecific findings of erythema and oedema.

CT scan is not required for diagnosing CDI, especially for mild-to-moderate disease, but it can be useful for recognizing more severe forms. Colonic inflammation can also be shown on CT

as increased thickening of the colonic wall (Ash et al., 2006) with trapping of contrast material, pancolitis, pericolonic fat changes and ascites.

1.7 Treatment of *Clostridium difficile* infection

Treatment should be based on disease severity or whether one is treating initial or recurrent CDI. In asymptomatic carriers, treatment is not indicated as available data suggest that treatment in these individuals would not prevent symptomatic transmission or infection (Goudarzi et al., 2014).

Metronidazole and oral vancomycin are recommended for the treatment of initial episode. Patients with mild-to-moderate CDI should be treated with metronidazole 500 mg orally 3 times a day for 10 days. Metronidazole has similar efficacy as vancomycin for treatment of mild to moderate CDI. Unlike vancomycin, metronidazole is well absorbed and its faecal concentration is very low or none in healthy volunteers and asymptomatic *C. difficile* carriage (Vecchio and Zacur, 2012, Khanna and Pardi, 2012). Routine use of vancomycin is not recommended due to the risk of development of vancomycin resistance in other organisms especially enterococci (Vecchio and Zacur, 2012, Khanna and Pardi, 2012, Apisarnthanarak et al., 2002). However failure to respond to metronidazole therapy within 5-7 days should prompt consideration of a change in therapy to vancomycin at standard dosing. (Musher et al. 2005, Surawicz et al. 2013). The time to resolution of diarrhoea might be shorter with vancomycin than with metronidazole therapy (Belmares et al. 2007).

Patients with severe CDI should be treated with vancomycin 125 mg orally four times per day for 10 days (Cohen et al., 2010). In treatment failure with low dose oral vancomycin or complicated CDI, it is recommended to use high-dose (500mg every 6 hours) oral vancomycin plus intravenous metronidazole, 500 mg 3 times a day (Khanna and Pardi, 2012, Cohen et al.,

2010). Administration of vancomycin via enema is used for patients with surgical or anatomic abnormalities.

Treatment of the first recurrence of CDI is the same as the treatment of first episode of CDI. In patients with a second recurrence of CDI, vancomycin should be the treatment of choice. Tapered or pulse-dosage vancomycin may reduce the risk of a subsequent recurrence (McFarland et al., 2002).

Fidaxomicin is a new macrocyclic that might be favoured over oral vancomycin in patients with multiple recurrences. Fidaxomicin can be applied for treatment of patients at high risk of recurrent CDI, patients infected with non-hypervirulent strain, patients with multiple episodes of recurrence, and patients who are not able to tolerate oral vancomycin (Khanna and Pardi, 2012, Knight and Surawicz, 2013).

Faecal microbiota transplantation (FMT) is an alternative therapy for treatment of recurrent cases of CDI. In this method, normal faecal microbiota in patients is restored using intestinal microorganisms from a healthy donor stool. FMT has high success rate of in treating CDI with rapid and enduring response with effectiveness of 92% of cases while the success rate of FMT via enema, nasogastric route, and colonoscopy was 95%, 76%, and 89%, respectively (Gough et al., 2011).

CHAPTER 2: STUDY OBJECTIVES

2.1 General Objectives

2.1.1 To explore the prevalence and its associated risk factors for *C. difficile* infection in hospitalised patients with loose stools or suspected cases in HUSM.

2.1.2 To explore the carriage rate and its associated risk factors for *C. difficile* among the elderly in the community from Tumpat and Kota Bharu district in Kelantan.

2.1.3 To determine the awareness of *C. difficile* infection among hospital staff and students in HUSM.

2.2 Specific Objectives

2.2.1 Hospital study

2.2.1.1 To determine the prevalence of *C. difficile* infection in hospitalised patients with loose stools or suspected cases in HUSM.

2.2.1.2 To determine the association between *C. difficile* infection and prior use of antibiotics.

2.2.1.3 To determine the ribotyping of *C. difficile* from culture positive stool samples of hospitalised patients.

2.2.1.4 To determine the initial clinical presentation in patients with *C. difficile* infection.

2.2.1.5 To determine the severity of disease and outcome in infected hospitalised patients.

2.2.2 **Community study**

2.2.2.1 To determine the carriage rate of *C. difficile* among the elderly in the community.

2.2.2.2 To determine the risk factors associated with *C. difficile* carriage.

2.2.2.3 To determine the ribotyping of *C. difficile* from culture positive stool samples of elderly in the community.

2.2.3 Awareness study

2.2.3.1 To determine the awareness among medical staff and students within HUSM with regards to *C. difficile* infection.

2.3 Research Questions

2.3.1 What is the prevalence of *C. difficile* infection in hospitalised patients in HUSM and the carriage rate in the elderly community in Kelantan?

2.3.2 Are there any associations between antibiotics and proton pump inhibitor exposure with the prevalence of *C. difficile* infection in HUSM and also *C. difficile* carriage in the community?

2.3.3 What are other associated factors in the prevalence of *C. difficile* infection or carriage?

2.3.4 What is the awareness among medical staff with regards to *C. difficile* infection?

CHAPTER 3: METHODOLOGY

3.1 Study Design

Hospital arm

This is a prospective cross sectional study among hospitalised patients in HUSM with *C. difficile* infection. Similar studies are also being conducted concurrently involving three other centres in Malaysia i.e. Universiti Kebangsaan Malaysia (UKM), Universiti Malaya (UM) and Universiti Teknologi Mara (UiTM) in collaboration with The University of Western Australia.

Community arm

This is a cross sectional study of *C. difficile* carrier prevalence among the elderly in the community in Kelantan.

Awareness Survey

This is a cross sectional study of *C. difficile* awareness among hospital staff (doctors, non-clinical medical professionals, paramedics) and students (postgraduate, undergraduate) in HUSM.

3.2 Study Location and Study Duration

Hospital study

This study was carried out in hospitalised C. *difficile* patients in HUSM from 1st April 2015 until 30th September 2015.