DIRECT DETECTION OF *Campylobacter* IN CHILDREN WITH LOOSE STOOL BY A DUPLEX POLYMERASE CHAIN REACTION (PCR)

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE DEGREE OF MASTER OF PATHOLOGY (MEDICAL MICROBIOLOGY)

SCHOOL OF MEDICAL SCIENCES UNIVERSITI SAINS MALAYSIA

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LIST OF SYMBOLS

Symbols/Abbreviations	Meaning
≤	Equal or less
>	More than
<	Less than
=	Equal
%	Percentage
µL	Microliter
˚C	Celsius
N	Frequency
hipO	Hippurate hydrolase
asp	Aspartokinase
HUSM	Hospital USM
SEA	South-East Asia
PCR	Polymerase Chain Reaction

HIV	Human immunodeficiency virus

ABSTRAK

Pengenalan: Cirit-birit adalah masalah seluruh dunia. *Campylobacter* adalah salah satu punca utama gastroenteritis di kedua-dua negara membangun dan negara maju. Dua spesies *Campylobacter* yang signifikan secara klinikal pada manusia ialah *Campylobacter jejuni* dan *Campylobacter coli*, yang merangkumi kira-kira 98% daripada semua gastroenteritis *Campylobacter* dalam manusia. Kajian ini bertujuan untuk mengenal pasti kekerapan *Campylobacter jejuni* dan *Campylobacter coli* pada kanak-kanak yang mengalami cirit birit dengan menggunakan PCR dupleks dan untuk mengkaji kaitan ciri demografi dengan pemerolehan *Campylobacter*.

Metodologi: Ini adalah kajian keratan rentas yang dijalankan di Makmal Mikrobiologi & Parasitologi Perubatan, Hospital USM. Sampel najis daripada kanak-kanak berumur ≤ 12 tahun yang dihantar untuk kultur dan memenuhi kriteria kajian dimasukkan dalam kajian. Kultur najis diproses mengikut piawai protokol makmal. Sisa sampel dianalisis menggunakan PCR dupleks konvensional yang menyasarkan gen *hipO* dan gen *asp* khusus untuk *Campylobacter jejuni* dan *Campylobacter coli.* Keputusan kultur dikumpulkan dari sistem maklumat makmal dan rekod perubatan telah disemak untuk ciri demografi pesakit.

Keputusan: Sebanyak 296 sampel najis tidak berulang yang memenuhi kriteria kajian telah dimasukkan dalam kajian. Daripada 296 sampel, 7.09% (n=21) adalah daripada jabatan pesakit luar dan baki 92.91% (n=275) adalah daripada jabatan pesakit dalam. Majoriti kes adalah kanak-kanak berumur 5 tahun ke bawah dengan purata umur 2.88 tahun. Daripada semua, 20.27% (n=60) adalah positif untuk *Campylobacter jejuni* dan/atau *Campylobacter coli* dengan menggunakan

PCR dupleks. Kultur berjaya mengasingkan 3.72% (n=11) *Campylobacter jejuni* dan/atau *Campylobacter coli.* Sampel positif kultur juga positif oleh PCR dupleks. Analisis seterusnya menunjukkan tiada kaitan yang signifikan antara umur, jantina, dan status imun dengan pemerolehan *Campylobacter.*

Kesimpulan: Sebagai kesimpulan, kekerapan jangkitan *Campylobacter* adalah lebih tinggi daripada jangkaan dan PCR mengesan lebih banyak kes yang terlepas dengan ketara oleh kultur rutin.

Kata kunci: *hipO* gene, *asp* gene, *Campylobacter jejuni*, *Campylobacter coli*

ABSTRACT

Introduction: Diarrhoea is a worldwide problem. *Campylobacter* is one of the major causes of gastroenteritis in both developing and developed worlds. The two *Campylobacter* species that are clinically significant in humans are *Campylobacter jejuni* and *Campylobacter coli*, which account for around 98 % of all human *Campylobacter* gastroenteritis. This study aims to determine the prevalence of *Campylobacter jejuni* and *Campylobacter coli* in children with loose stool using a duplex PCR and to study the association of demographic characteristics with acquisition of *Campylobacter*.

Materials and methods: This is a cross sectional study conducted in Medical Microbiology & Parasitology Laboratory, Hospital USM. Stool samples from children ≤ 12 years old sent for culture and met the inclusion criteria were included in the study. Stool cultures were processed according to the standard laboratory protocols. Leftover samples were analyzed using a conventional duplex PCR targeting *hipO* gene and *asp* gene specific for *Campylobacter jejuni* and *Campylobacter coli*. Culture results were gathered from laboratory information system and medical records were reviewed for demographic characteristics of the patients.

Results: A total of 296 non-duplicate stool samples that fulfilled the inclusion criteria were included in the study. From 296 samples, 7.09% (n=21) were from outpatient department and the remaining 92.91% (n=275) were from inpatient department. Majority of cases were children under 5 years old with the mean age of 2.88 years old. Of all, 20.27% (n=60) were positive for *Campylobacter jejuni* and/or *Campylobacter coli* using the duplex PCR. Cultures were successfully isolated 3.72% (n=11) *Campylobacter jejuni* and/or *Campylobacter coli*. Samples

with positive culture were also positive by the duplex PCR. Subsequent analysis showed no significant association between age, gender, and immune status with *Campylobacter* acquisition.

Conclusion: As a conclusion, prevalence of *Campylobacter* infection was higher than expected and PCR detected more cases which significantly missed by routine culture.

Keyword: *hipO* gene, *asp* gene, *Campylobacter jejuni*, *Campylobacter coli*

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Diarrhoea is an acknowledged issue worldwide. In 2016, an estimated 1.6 million fatalities worldwide are caused by diarrhoea, which is the eighth leading cause of death in all age groups (Troeger *et al.*, 2018). The most frequent causative agents of diarrhoea include bacteria such as *Salmonella* spp., *Vibrio cholerae*, *Campylobacter jejuni*, *Escherichia coli*, *Aeromonas* spp., and *Yersinia enterocolitica*; viruses largely rotavirus, adenovirus, and norovirus; protozoa mainly *Entamoeba histolytica*, *Giardia* spp. and *Cryptosporidium* spp (Gahamanyi *et al.*, 2020). Among the bacterial aetiologies, *Campylobacter* is the main cause of gastroenteritis in both developing and developed countries (Igwaran & Okoh, 2019).

Two *Campylobacter* species that are clinically significant in humans are *Campylobacter jejuni* and *Campylobacter coli*, which account for around 98% of all human *Campylobacter* gastroenteritis. Even though *C. coli* has lower prevalence compared to *C. jejuni* in certain geographical areas, *C. coli* can nevertheless account up to a quarter of all *Campylobacter* gastroenteritis cases (Kaakoush *et al.*, 2015). Symptoms of *C. jejuni* and *C. coli* infection include acute watery or bloody diarrhoea, fever, weight loss, and abdominal pain that linger for an average

of 6 days. Gastroenteritis induced by *C. jejuni* is indiscernible from that of *C. coli* (Kaakoush *et al.*, 2015).

Conventional culture is the most widely method being used in clinical microbiology laboratories for the detection of *Campylobacter*. However, not only it takes a lot of time (usually up to 4 days), but the culture media and conditions can impede the isolation of some *Campylobacter* species. Due to the challenges associated with the regular method of *Campylobacter* detection, this makes *Campylobacter* as excellent candidates for identification by polymerase chain reaction (PCR).

Few studies were conducted to discover the prevalence of *Campylobacter* in Malaysia. However, most of the studies are based on poultry samples and till date, in our country, there is very few studies on prevalence of *Campylobacter* isolated from human sample which used conventional culture method. To the best of our knowledge, to date (as of November 2022), there are no publications on the detection of *Campylobacter* in human using PCR in Malaysia. The goal of this study was to describe the prevalence of *Campylobacter* in human, particularly children by PCR analysis, which is more rapid, sensitive and specific compared to conventional methods.

1.2 Literature review

1.2.1 *Campylobacter*

Campylobacter was first being described in 1957 owing to the isolation of related *Vibrio* from blood samples in children with loose stool. In the 1970s, stool samples could be tested in clinical laboratories for *Campylobacter* due to the invention of selective growth medium (Altekruse *et al.*,

1999). Following the establishment of simple culture methods, by the early 1980s, they had become well-known as important cause of bacterial gastroenteritis (Altekruse *et al.*, 1999; Newell *et al.*, 2017).

The *Campylobacter* genus is a member of the family *Campylobactereceae*, the order *Campylobacterelas*, the class *Epsilonproteobacteria*, and the phylum *Proteobacteria*. The *Campylobacter* genus was created subsequently after the renaming of *Vibrio foetus* to *Campylobacter foetus* in 1963, constituting the type species of this genus. Since its first identification, the genus has expanded to comprise several significant human and animal infections that are mostly categorized using phylogenetic methods (Kaakoush *et al.*, 2015). Currently, the genus *Campylobacter* consists of more than 30 distinct species (as of November 2022).

Campylobacter species are slender Gram-negative curved, spiral or rod-shaped non spore forming microaerophilic bacteria. Some *Campylobacter* species have a single polar flagellum, while some might have bipolar flagella, or no flagellum at all depending on the species (Okoli, Wadstrom & Mendz, 2007; Man, 2011). *Campylobacter* species has characteristics of indole negative, oxidase positive, Hippurate positive, catalase positive, nitrate positive and glucose utilization negative (Linton, Owen & Stanley, 1996; Igwaran & Okoh, 2019).

Campylobacter is fastidious and microaerophilic organisms that able to grow at temperature between 37°C to 42°C. At low temperature, the bacteria can survive and carry out essential biological functions including protein synthesis, however growth and recovery is challenging (Davis & DiRita, 2008). Because *Campylobacter* is fastidious organisms that need microaerobic conditions to flourish, the techniques utilized for collecting, transporting, and culturing stool samples might significantly affect the test's sensitivity (Hurd *et al.*, 2012).

1.2.2 Transmission routes of *Campylobacter* infection

Animals are typically thought as hosts or reservoirs and the environment and water as possible sources. The mode of transmission of *Campylobacter* infection in human are through ingestion of contaminated food (especially poultry origin) or water or through direct contact with animals (Allos, 2001; Huang *et al.*, 2009; Whiley *et al.*, 2013; Newell *et al.*, 2017; Tresse, Alvarez-Ordóñez & Connerton, 2017). Thus, those who had swimming in rivers, consumption, and handling of chicken meat, are at risk of getting *Campylobacter* infection. Nevertheless, handling, preparing, and consuming contaminated food is believed to be the primary mode of transmission of *Campylobacter* in humans.

There are published reports regarding similarities and overlapping between the serotypes of *C. jejuni* identified in human, poultry, and cattle. These findings showed that eating animals' product may be a significant factor in the transmission of *C. jejuni* to humans (Nielsen, Engberg & Madsen, 1997; Padungtod & Kaneene, 2005). Poultry intestines are easily colonized by *C. jejuni* and most of them are colonized by the age of four weeks (Kapperud *et al.*, 1993). Few reservoirs were identified in the poultry environment which include unchlorinated drinking water (Snelling *et al.*, 2005), and farm workers (Kapperud *et al.*, 1993).

1.2.3 *Campylobacter* infection

There are not many publications on the *Campylobacter* infective dosage. One study mentioned that infection with *C. jejuni* or *C. coli* at oral dose of 500 colony forming unit (CFU) might lead to human disease (D A Robinson, 1981). Nevertheless, report has shown that ingestion dose of

Campylobacter as low as 360 CFU has been associated with human infection (Hara-Kudo & Takatori, 2011). On the other hand, it is widely known that repeated exposures (at low doses) to *Campylobacter*, can result in formation of specific immunity which is adequate to provide defense against clinically overt disease, but not always be effective against colonization. This condition explains why it is uncommon for adults in the developing world to acquire *Campylobacter* infection (Janssen *et al.*, 2008; Newell *et al.*, 2017).

Campylobacter mainly causes infections in the gastrointestinal tract. Sometimes it is impossible to distinguish between *Campylobacter* with *Shigella* and *Salmonella* based on their clinical symptoms alone (Allos, 2001; Igwaran & Okoh, 2019).

Campylobacter infection has incubation period of two to five days. Most patients experience three to seven days of watery diarrhoea (occasionally bloody diarrhoea) along with nausea, vomiting, abdominal cramp, fever and headache. Most *Campylobacter* infections are self-limiting within a week, but they can remain longer in some patients, and they can even last up to three weeks in about 20% of patients. In very few occasions, patients might present with longer, relapsing diarrhoea illness that persists for weeks (Kaakoush *et al.*, 2015; Hansson *et al.*, 2018). While gastroenteritis is a main clinical condition resulting from *Campylobacter* infection, these organisms have also been linked to a number of other serious conditions within the gastrointestinal tract, such as inflammatory bowel diseases (IBD), celiac disease, esophageal diseases, functional gastrointestinal disorders, cholecystitis, periodontitis, and colon cancer (Kaakoush *et al.*, 2015).

Extra-gastrointestinal infection also may occur in *Campylobacter* infection, but less common. Extra-gastrointestinal involvement may be manifested as bacteremia, pneumonia, brain abscess, meningitis, osteomyelitis, and septic arthritis. Guillain-Barré syndrome (GBS) is the most

significant post-infectious manifestation following *Campylobacter* infection. Despite its rarity, GBS is regarded as a very serious condition since it can lead to substantial neurological sequelae and, in some cases, a fatality rate of 2–3 percent (Hansson *et al.*, 2018; Igwaran & Okoh, 2019).

Most *Campylobacter* infections are self-limiting and do not need any treatment beyond supportive measure, like hydration therapy and correction of electrolyte imbalance. However, antibiotics are administered in patients with symptoms prolonged or severe, immunocompromised, and those with extra-gastrointestinal infections (Martin *et al.*, 2008; Sproston, Wimalarathna & Sheppard, 2018).

1.2.4 The epidemiology of *Campylobacter*

While infection of *Campylobacter* can occur in patients of various ages, it occurs mainly in extreme age population includes both infants and elderly (Engberg *et al.*, 2000). In the United States, it has been reported that *Campylobacter* infection frequently occurs in children younger than 5 years of age (Same & Tamma, 2018). In another study conducted in China revealed that there is no significant difference between genders and age of *Campylobacter* positive cases (Li *et al.*, 2018). In addition to that, patients who have human immunodeficiency virus (HIV) infections as well as those who are immunocompromised, such as those with primary immunodeficiency and extreme prematurity <32 weeks are more vulnerable to *Campylobacter* infections (Ben-Shimol, Carmi & Greenberg, 2013; Kim *et al.*, 2017).

1.2.4 (a)Worldwide

The epidemiology of *Campylobacter* infections seemed to be discrete between developing and developed countries. In developed countries, symptomatic infection seen in various age groups, while in developing countries, majority of *Campylobacter* infections are found in young children, and infection in adults is thought to be non-symptomatic (Osbjer *et al.*, 2016). The key sources of human *Campylobacter* infections in developed countries include ingestion of chicken meat, drinking contaminated water, consumption of unpasteurized dairy products, and direct contact with farm animals (Domingues *et al.*, 2012; Food, Authority & Centre, 2015). Information on source attribution in developing countries are insufficient, but sources include lack of sanitary, raising animals at home, and consumption of contaminated water (Rao *et al.*, 2001; El-Tras *et al.*, 2015).

Campylobacter was listed as the most frequently reported gastrointestinal bacterial pathogen in humans in the European Union (EU) and has been so since 2005, according to a summary report by the European Union on trends and etiologies of zoonoses, zoonotic agents, and food-borne outbreaks in 2015 (Food & Authority, 2015). *Campylobacter* was the most prevalent bacterial cause of gastroenteritis in Australia, New Zealand, and the United States (Hansson *et al.*, 2018). Other countries of the world were also plagued by a serious issue with campylobacteriosis (Kaakoush *et al.*, 2015).

Prevalence varies among countries, basically depends on many factors such as studied population and detection methods used. A comparative analysis of culture and PCR based assays for the detection of *Campylobacter* species in human stool samples had also been carried out in India. The results demonstrated differences between traditional culture and advanced molecular

methods. Out of 75 samples, 6.6% were positive by PCR, but only 1.33% were positive by culture. Furthermore, the samples that were positive by culture also tested positive by PCR (Pallavi, Kumar & Kumar, 2015).

In Cambodia, a study was conducted to detect *Campylobacter* in human stool samples by PCR and culture method. The observed prevalence of *C. jejuni* and *C. coli* in children using PCR method was 19%, while none were tested positive by culture method although the duplicate samples were processed for culture in different clinical laboratories applying the standard protocol used for detection of *Campylobacter* (Osbjer *et al.*, 2016). Another study in Thailand reported 17.6% prevalence of *Campylobacter* among hospitalized patients with diarrhoea using PCR method (Padungtod & Kaneene, 2005).

1.2.4 (b) Malaysia

Several studies conducted in Malaysia have investigated the prevalence of *Campylobacter* in animals particularly poultry and pets. A study in Selangor by Selim *et al* reported that 20% of *Campylobacter* were detected in chicken meat by PCR method (Selim *et al.*, 2013). Another study in Selangor revealed that the prevalence of *Campylobacter* in cats and dogs using PCR testing were 23.25% and 14.85% respectively (Jalo, Aung & Mohamed, 2015). Recent unpublished study by Muhammad conducted in Kelantan reported that 22% of *Campylobacter* were detected by PCR in retail broiler chicken, while another study done in Kelantan showed prevalence of 20% *Campylobacters* by PCR among the living broilers (Nur-Aziera-Aina, Nasir & Zaidah, 2020).

On the other hand, there has been very few studies have been conducted to determine the prevalence of *Campylobacter* from human samples in Malaysia. According to a previous study conducted in Kuala Lumpur from 1983-1984, *C. jejuni* was recovered from stool samples by using traditional culture method from 3.8% of children and from 4.3% of adult with diarrhoea. Though *C. jejuni* were not apparent in adult without diarrhoea, the isolation rate was approximately 2.6% in children control group (Lim, Jegathesan & Wong, 1984). More recent study in hospital centres in Malaysia involving Kuala Lumpur and Johor Bharu investigated the enteropathogens in children aged less than 14 years old with acute diarrhoea in 2002. Based on this study, *Campylobacter* was found as one of the top five most common isolated bacteria pathogens, which were recovered by culture method (Lee *et al.*, 2006).

1.2.5 Laboratory diagnosis of *Campylobacter*

The application of culture-dependent and/or culture-independent methods is required for the laboratory diagnosis of *Campylobacte*r infection. Culture is known as the "gold standard' for detection of *Campylobacter* infection (de Boer *et al.*, 2015). For culture dependent method, single isolated colonies can be subjected to a range of traditional biochemical test to recognize the phenotypic traits. In order to identify the organism to the genus or species level, the biochemical profile of unknown organism is matched to previously defined characteristics of the *Campylobacter* genus or species (Kaakoush, Mitchell & Man, 2014). However, the major disadvantage of the current gold standard for diagnosis of *Campylobacter* is time consuming (de Boer *et al.*, 2015). Furthermore, stool culture has long been thought to miss a considerable amount of *Campylobacter* infections detection, despite its lengthy history of usage in clinical laboratories.

Buss *et al.* conducted a large prospective study and concluded that *Campylobacter* culture testing had poor sensitivity. This is in agreement with previous study done before on sensitivity of *Campylobacter* culture. The study reported that around 25% of the specimens that were proven to have *Campylobacter* DNA and bacterial antigen had been labelled as culture-negative (Buss *et al.*, 2019).

Culture-independent methods including enzyme immunoassay (EIA)-based stool antigen tests and nucleic acid-based tests have been invented since the last decades (Platts-Mills & Kosek, 2014). Early research revealed that antigen tests had outstanding sensitivity and specificity when compared to culture of children and adults in developed countries (Granato *et al.*, 2010). However, some recent investigations have indicated a significant false-positivity rate of *Campylobacter* with routine use in clinical microbiology laboratories (Bessède *et al.*, 2011; Giltner *et al.*, 2013).

The usage of polymerase chain reaction (PCR) was first being introduced in 1992 in specific identification of *C. jejuni* and *C.coli* (Oyofo *et al.*, 1992). PCR is broadly used for an identification of *Campylobacter* up to species level (Banowary *et al.*, 2015). DNA or RNA can be extracted using a molecular technique from pure cultures or clinical samples. The genetic marker of the organism can then be identified using a sequencing method, or genus- or species-specific PCR amplification of the gene of interest (Kaakoush *et al.*, 2015). Over the years, molecular methods have been constructed to provide better diagnostic techniques for detection of *Campylobacter* (Ricke *et al.*, 2019).

1.3 Rationale of the study

Assessment of the effects of human *Campylobacter* infection in the South-East Asia (SEA) region is hampered by a lack of information on morbidity, death, and related consequences. Reliable baseline data backed by the national monitoring system are needed to get an insight into *Campylobacter* infection in a nation. Thus, studies on the epidemiology and prevalence of *Campylobacter* are necessary for a better understanding of the disease with regard to source of infection, risk factors, disease transmission, severity, and associated sequalae as well as to eventually create a population-level intervention approach.

In Malaysia, a number of studies were already carried out in livestocks, particularly poultry such as chickens and ducks, as well as on poultry meat and wild birds. However, there are very few data regarding *Campylobacter* infection in human. By using PCR method, a rapid, sensitive, and specific method for detection of *C. jejuni* and *C. coli* would be beneficial in reducing the time of diagnosis and treatment.

1.4 Research question

1. What is the prevalence of *Campylobacter* in children with loose stool in HUSM?
2. What is the detection rate of *Campylobacter jejuni* and *Campylobacter coli* by using duplex PCR compared to culture method?
3. What is the association between demographic characteristics of patients with loose stool and acquisition of campylobacteriosis?

1.5 Research hypothesis

1. The prevalence of *Campylobacter* in children with loose stool in HUSM is comparable to the prevalence elsewhere in SEA region.
2. PCR method is superior to culture method in detection of *Campylobacter*.
3. There is association between demographic characteristics of patients with loose stool and acquisition of campylobacteriosis.

1.6 Study objectives

General Objective

To detect *Campylobacter jejuni* and *Campylobacter coli* by duplex PCR in children with loose stool in Hospital USM (HUSM)

Specific objectives

1. To describe the proportion of *Campylobacter jejuni* and *Campylobacter coli* in children with loose stool in HUSM by duplex PCR.
2. To describe the detection rate of *Campylobacter jejuni* and *Campylobacter coli* by duplex PCR and routine stool culture.
3. To determine the association between demographic characteristics of patients with loose stool and acquisition of campylobacteriosis.

1.7 Study flowchart

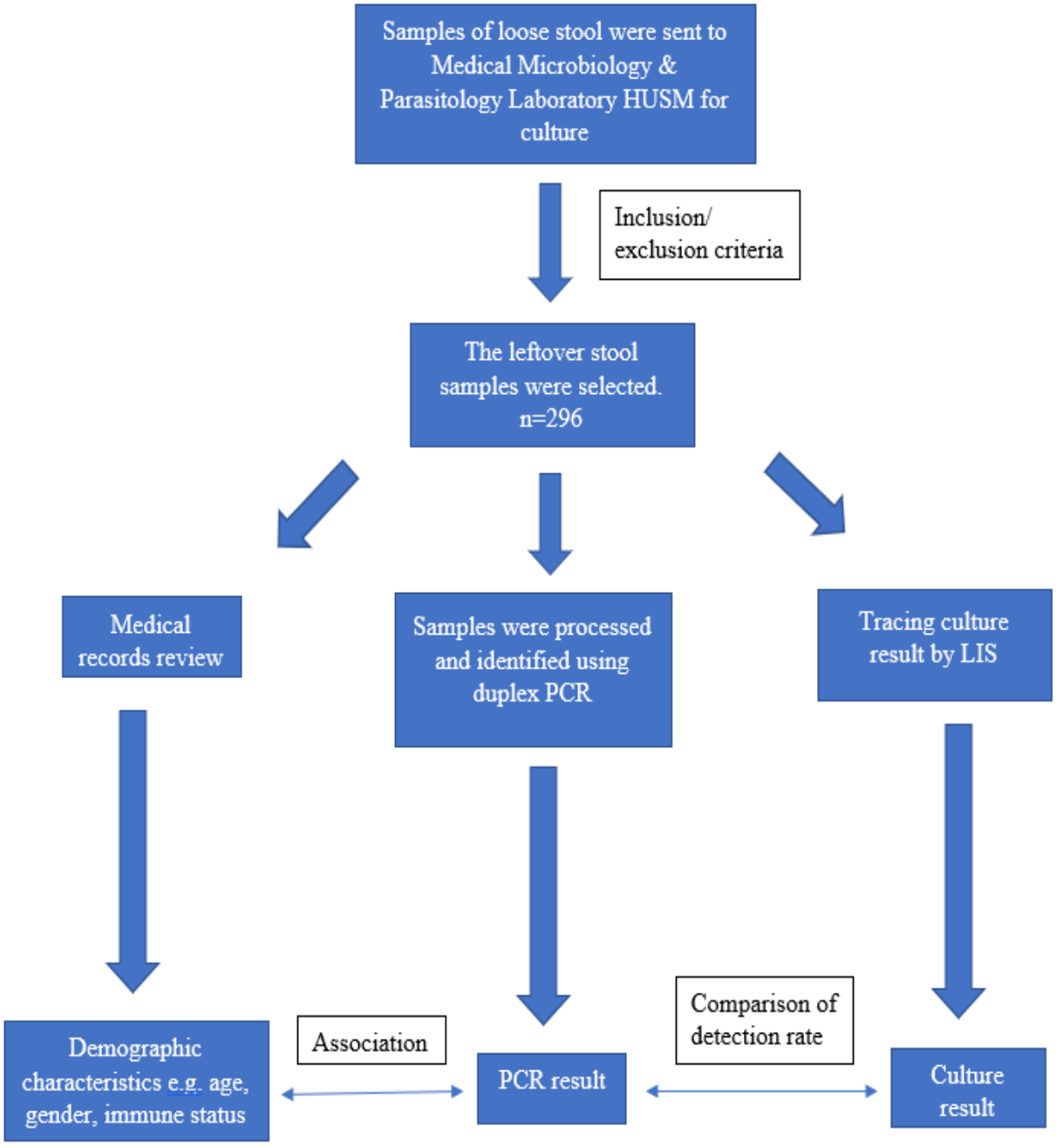

Figure 1: Flow chart of the study

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CHAPTER 2

STUDY PROTOCOL

The content of this study protocol has been submitted earlier for ethics approval by the Universiti Sains Malaysia Human Research Ethics Committee (JEPeM-USM).

2.1 Title

Direct Detection of *Campylobacter* in children with loose stool by a duplex Polymerase Chain Reaction (PCR).

2.2 Objective

2.2.1 General objective

To detect *Campylobacter jejuni* and *Campylobacter coli* in children with loose stool in Hospital USM (HUSM) by a duplex PCR.

2.2.2 Specific objectives

1. To describe the proportion of *Campylobacter jejuni* and *Campylobacter coli* in children with loose stool in HUSM by duplex PCR.
2. To describe the detection rate of *Campylobacter jejuni* and *Campylobacter coli* by duplex PCR and routine stool culture.

3. To determine the associated factors in children with loose stool with acquisition of campylobacteriosis.

2.3 Methodology

2.3.1 Study design

This is a cross sectional study conducted in major hospital with clinical microbiologists in Hospital USM, Kubang Kerian, Kelantan from early April 2021 until end of May 2022.

2.3.2 Reference population

All loose stool samples from children ≤ 12-year-old submitted to Medical Microbiology & Parasitology Laboratory for culture from inpatient and outpatient departments in Hospital USM.

2.3.3 Target Population

All loose stool samples from children ≤ 12-year-old submitted to Medical Microbiology & Parasitology Laboratory for culture from inpatient and outpatient departments in Hospital USM.

2.3.4 Source population

All loose stool samples from children ≤ 12-year-old submitted to Medical Microbiology & Parasitology Laboratory for culture from inpatient and outpatient departments in Hospital USM from April 2021 until May 2022.