

Characterization of house fly microbiota and antibiotic resistant bacterial species they are harbouring

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ABSTRACT

Housefly (*Musca domestica* L.) is an insect belonging to the order Diptera, family Muscidae. This insect can frequently carry bacteria in their digestive tract or on the body surface. Accurate identification of houseflies is an essential factor for both epidemiological studies and the precise formulation of vector control strategies. Thus, numerous publications have reported on pathogenic bacteria including those of antimicrobial resistant isolated from housefly samples. Houseflies are highly mobile and can spread pathogenic bacteria by mechanical contact with the substrates and the habitat of houseflies harbours a diverse microbial community. The rapid adaptation process of ingested microbes in the insect gut may involve gene transfer including antibiotic resistant determinants among different bacterial strains. Hence, the aim of this study was to determine the occurrence and characterize houseflies sampled from the hospices and to detect microbial pathogens together with their associated resistant genes of medical and veterinary importance that they are harbouring.

A systemic review and meta-analysis were conducted using published articles from 1980 to 2020 retrieved from electronic databases assessed for eligibility according to Preferred Reporting Items for Systemic Reviews and Meta-Analysis guidelines. Seventy-eight studies were included in the review with only 44 studies eligible for meta-analysis with only five pathogenic bacterial species including *Escherichia coli*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* being focused on for meta-analysis. Houseflies collected from hospices were identified using published species identification keys to confirm the observed features and to supplement the morphological observation, PCR targeting the *16S rRNA*, *CO1* and *ITS2* genes were conducted to identify and characterize houseflies to species level. The bacterial communities harboured by houseflies were determined using NGS platform, whilst the antibiotic resistance and virulence genes from microbial communities associated with houseflies were detected by PCR assays.

The systematic review revealed that houseflies carried a considerable number of susceptible and antibiotic-resistant bacteria, including multiple-antibiotic resistant strains. Majority of studies have used culture methods for the detection of bacteria from houseflies. A total of

657 houseflies were collected comprising of 306 (46.6%), 258 (39.3%) and 93 (14.2%) from Sparrow hospice, Lebone hospice, and from Sunflower hospice respectively. The morphological identification of houseflies was successful and further supported by amplification of *16S rRNA*, *CO1* as well as *ITS2* gene fragments. Generated sequences for the targeted gene fragments matched with *Musca domestica* and all sequences were deposited to the GenBank database. The microbiota composition at the phylum, class, order and genus level were assessed. The most abundant phyla detected were Proteobacteria, followed by Firmicutes and Bacteroidetes. Multiple species of bacteria were identified to genus level present in some housefly samples from some hospices. The samples from Lebone and Sparrow hospice were significantly more diverse as compared to those from Sunflower hospice. The tested housefly samples shared some of the bacterial species, if not all. Antibiotic resistant genes detected from the housefly DNA in this study included *ermB*, *tetA*, *blaSHV* and *blaTEM* genes associated to resistance for erythromycin, tetracycline, and beta-lactams antibiotics, respectively. There were no virulence genes detected for the selected bacterial pathogens from housefly samples in all three hospices investigated.

The systematic review and meta-analysis have provided global overview on the carriage of considerable bacterial pathogens including antibiotic resistant bacteria detected in houseflies from different regions of the world. The identification of housefly by morphological and molecular identification in this study, confirmed the importance of considering both techniques for identification of houseflies. The application of DNA barcoding can delineate the limitations between species and allocate taxonomic status to unidentified species from known species. Generated data from this study showed that houseflies collected from different hospices are associated with highly diverse microbial communities and some with antibiotic resistant genes detected. As such, this insect poses tremendous health risk in hospices and surrounding communities since they are potential vector of pathogenic microorganisms. It is recommended that there should be formulation of vector control strategy to ensure that houseflies are controlled both inside and outside the hospice facility. There should be continuous education on the proper use of antibiotics especially at the human care centres. Furthermore, there is need to do further researcher for genotyping the bacteria of medical importance carried by the hospice houseflies.

Key words: Housefly, Hospice, Microbial communities, *Musca domestica*, antibiotic resistance, bacterial pathogens

DEDICATION

I dedicate this study to my family

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RESEARCH OUTPUTS

Full-length article

Monyama MC, Onyiche ET, Taioe MO, Nkhebenyane JS, Thekiso OMM. Bacterial pathogens identified from houseflies in different human and animal settings: A systematic review and meta- analysis. *Veterinary Medicine and Science*. 2022; 8(2):827–844. <https://doi.org/10.1002/vms3.496> (Appendix A).

Conference presentation

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CHAPTER 1

GENERAL INTRODUCTION

1.1 BACKGROUND

There are approximately 18 000 species of true flies (Diptera), and among them are those of medical and veterinary importance belonging to families such as Calliphoridae, Ceratopogonidae, Culicidae, Glossinidae, Muscidae, Fanniidae, Simuliidae as well as Tabanidae (Service, 2012). Dipteran flies are one of the most abundant and important groups of insects, serving as vectors for some of the most devastating diseases affecting animals and humans (Gupta *et al.*, 2012). The family Muscidae comprises approximately 4 200 fly species belonging to 190 genera (Service, 2012).

The most well-known members from Muscidae are commonly referred to as houseflies, *Musca domestica* in particular (Gupta *et al.*, 2012; Nihei and De Carvalho, 2007). The adult *M. domestica* regularly avoids direct sunlight, preferring to shelter in buildings inhabited by people or animals (Service, 2012). Houseflies are important nuisance pests associated with humans and domesticated animals (Zhu *et al.*, 2016), as well as vectors of numerous foodborne and animal pathogens (Barreiro *et al.*, 2013). These pathogens range from viruses, bacteria, fungi, protozoa, and nematodes (Gupta *et al.*, 2012).

The habits and feeding mechanisms of houseflies enable them to spread bacteria and other disease-causing microorganisms (Bahrndorff *et al.*, 2017). As such, there is a complex relationship between houseflies and microorganisms, which has resulted in the formation of biological systems within which houseflies act as a biological or mechanical vector for various infectious agents (Rodhain, 2015). Houseflies transport diverse and active microbial communities from different environmental reservoirs (Barreiro *et al.*, 2013; Gupta *et al.*, 2012). Therefore, the risk of houseflies spreading resistant bacterial strains from livestock and poultry farms to the public is of great concern (Zhang *et al.*, 2018).

Houseflies colonized with bacterial species could as well be associated with the spreading of antibiotic-resistant genes or exposure of virulent bacterial strains within the same setting (Bouamama *et al.*, 2010; Zhang *et al.*, 2018). However, despite the complex

association, houseflies have rarely been implicated in any specific medical condition (Junqueira *et al.*, 2017).

Current techniques for Muscidae identification and classification involve the use of morphological keys and microscopic analysis, based on morphological features of the housefly including thorax, leg chaetotaxy and wing venation (Catts and Goff., 1992; Grzywacz *et al.*, 2017). Additionally, the advent of DNA-based methods has enabled genotypic analysis using mitochondrial DNA targeting the *CO1* gene as a molecular barcode used universally for insect identification and the use of internal transcribed spacer 2 (*ITS2*) gene as a second barcoding locus for taxonomic species identifications (GilArriortua *et al.*, 2014; Gutierrez *et al.*, 2014; Rotty *et al.*, 2018). The amplification of the 16S ribosomal RNA (*16S rRNA*) gene by PCR technique is regarded as an effective morphological method to identify Muscidae species (Li *et al.*, 2010).

The microbiological culture method is one of the most broadly used tools for microbiological identification and is considered a 'gold standard' because of its ability to detect new bacterial species and to test their susceptibility to antibiotic agents (Kotsilkov *et al.*, 2015). However, many bacteria are uncultivable, are difficult to isolate, or grow slowly in the culture due to stringent growth requirements or may not grow because of prior empirical treatment with antimicrobial agents (Muthukumar *et al.*, 2008; Zhao *et al.*, 2017). In contrast to long-established culture-based diagnostic methods, numerous culture-independent techniques, including checkerboard DNA–DNA hybridization, *in situ* hybridization, or polymerase chain reaction (PCR), have been applied to overcome the challenges imposed by the cultivation method. These molecular techniques concerning PCR, provide the means for rapid, specific, and sensitive detection of pathogenic bacteria and enable the examination of a much larger set of taxa (Kotsilkov *et al.*, 2015).

High-throughput DNA sequencing methods bring innovative opportunities to characterize bacterial communities (Zhao *et al.*, 2017). This brings about identifying both cultivable and uncultivable bacteria. This is accomplished to offer an extended perspective on bacterial diversity with higher coverage and a focus on a different set of organisms (Zhao *et al.*, 2017). The *16S rRNA* gene remains the standard tool for taxonomic identification (Park and Won, 2018). This is due to the reason that, existing microbial taxonomies based

on genomes have numerous limitations, including low phylogenetic resolution and lack of absolute numbers (Park and Won, 2018). Insect gut harbour an immense number of unculturable bacteria. Therefore, high throughput technologies with the amplification of the bacterial *16S rRNA* gene has been successfully applied to analyse various insect's gut microbiomes (Hadapad *et al.*, 2019). This culture-independent amplicon sequencing of the *16S rRNA* gene allows higher resolution compared to earlier studies addressing the microbiota of houseflies (Bahrndorff *et al.*, 2017). Therefore, this approach offers a comprehensive survey of the entire microbiota associated with the major synanthropic vector of pathogenic bacteria (Bahrndorff *et al.*, 2017).

Many studies focused more on houseflies collected from different locations including households, restaurants, hospitals, farms to mention a few (Issa, 2019). This study focused on the housefly samples collected from hospices. A hospice in South African context is defined as a non-governmental public organization that provides palliative care to terminally ill patients (Nkhebenyane and Lues, 2020). According to Rosenberg *et al.*, (2013) patients that are under hospice and palliative care are at high risk of bacterial infections. This is due to the potential risk imposed by food contamination that may occur in the kitchen during food preparation and storage (Nkhebenyane and Lues, 2020), thus houseflies are responsible suspects for the contamination of food.

The current study sought to determine the occurrence and characterize houseflies sampled from the hospices in the Bloemfontein and Johannesburg cities of South Africa. Furthermore, the study documented bacterial communities harboured by these houseflies using metabarcoding on the MiSeq next-generation sequencing (NGS) platform and also determined the antibiotic resistance and virulence genes in microbial communities associated with houseflies by species-specific PCR assays.

1.2 JUSTIFICATION

Pathogenic bacteria pose serious threats to the health of humans, wildlife, and crops. The spread of diseases in many cases is mediated through arthropod vectors (Rodhain, 2015). Muscoid flies have been identified as efficient biological vectors of human pathogens and are well known as carriers of disease-causing microorganisms (Bahrndorff *et al.*, 2017). South Africa has more than sixty registered hospices linked to

the Hospice and Palliative Care Association of South Africa [HPCA] (Nkhebenyane and Lues, 2013). However, there is no information available on the role of houseflies in the transmission/spread of pathogens in these institutions. One of the reasons for the lack of research on this topic is the laborious or limited nature of techniques that were previously used. Application of these techniques focused on culture-dependent assays that only employs culture alone or culture followed by PCR; focusing on limited or single species (Nayduch *et al.*, 2002; Alam and Zurek, 2004; Szalanski *et al.*, 2004, Gupta *et al.*, 2012).

The advent of DNA based technology has made it possible for development of various diagnostic assays which are reliable, specific and sensitive. The *16S rRNA* metagenomic approach allows the simultaneous sequencing of different DNA fragments while delivering accurate identification results (Abayasekara *et al.*, 2017). Therefore, this approach enables the scientific community as well as both human and animal health sectors to have a better understanding of the role played by gut microbes found in houseflies in the transmission of disease-causing bacterial pathogens. As a result, this study sought to contribute to filling in the information gap on the occurrence of various bacterial species in these public centers due to the presence of houseflies. The following hypotheses were formulated to address some of these knowledge gaps about houseflies in South Africa.

1.3 HYPOTHESES

- Previous published studies from around the world report on vectoral potential of houseflies in the transmission of bacterial pathogens.
- There is a variation in the abundance of houseflies from the sampled hospices in the different provinces due to differences in climate and vegetation.
- Houseflies (Muscidae) from hospices in South Africa are linked to a variety of bacterial communities.
- Houseflies are potential mechanical or biological vectors for the dissemination of virulent and antibiotic-resistant bacteria.

1.4 AIM AND OBJECTIVES

1.4.1 Aim

To determine the occurrence and characterize houseflies sampled from the hospices and to detect microbial pathogens together with their associated resistant genes of medical and veterinary importance that they are harbouring.

1.4.2 Objectives

- i. To conduct a systematic review and meta-analysis on bacterial pathogens identified from houseflies in different human and animal settings.
- ii. To conduct morphological and genetic characterization of houseflies (*Musca domestica*) from hospices in Bloemfontein and Johannesburg by amplification of *16S rRNA*, *COI* and *ITS2* and subject them to phylogenetic analysis.
- iii. To determine the gut microbiota of houseflies (*Musca domestica*) collected from hospices in Bloemfontein and Johannesburg by *16S rRNA* metagenomic analysis using next-generation sequencing.
- iv. To detect antibiotic resistance and virulence genes of bacteria from housefly (*Musca domestica*) DNA by species-specific PCR assays.

1.5 STRUCTURE OF THE THESIS

CHAPTER 1: GENERAL INTRODUCTION

Includes the background, justification, hypotheses, aim and objectives of the study.

CHAPTER 2: LITERATURE REVIEW

Provides literature review on the existing knowledge relating to house flies and their bacterial communities, as well as the development of antibiotic resistance from bacteria of public health importance.

CHAPTER 3: SYSTEMATIC REVIEW AND META-ANALYSIS OF BACTERIA HARBOURED BY HOUSEFLIES

This chapter is a systematic review and meta-analysis of previously published articles on bacterial pathogens identified from houseflies in different human and animal settings.

CHAPTER 4: IDENTIFICATION OF HOUSEFLY SPECIES INHABITING SAMPLED HOSPICES

Reports on morphological and genetic characterization of houseflies (Muscidae) collected from hospices in this study.

CHAPTER 5: DETECTION OF GUT MICROBIAL COMMUNITIES ASSOCIATED WITH HOSPICE HOUSEFLIES

This chapter reports comparative metagenomic analysis of bacterial communities found between different specimens of houseflies using next-generation sequencing.

CHAPTER 6: DETECTION OF ANTIBIOTIC RESISTANCE AND VIRULENCE GENES FROM BACTERIA HARBOURED BY HOUSEFLIES

This chapter reports on the detection of bacterial resistance and virulence genes from bacterial species of public health importance harboured by houseflies from hospices.

CHAPTER 7: CONCLUSION AND RECOMMENDATIONS

Includes concluding remarks on the findings of the entire project. Furthermore, the chapter provides recommendations for future research based on the observations of this study.

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CHAPTER 2

LITERATURE REVIEW

2.1 DIPTERAN FLIES

The order Diptera of class Insecta is comprised of true or two-winged flies (Alikhan *et al.*, 2016). Dipteran flies are the most abundant and important group of insects, serving as vectors for some of the most devastating diseases that affect humans and animals (Gupta *et al.*, 2012). There are 108 families of dipterans containing over 120,000 species, of which approximately 350 flies from 29 families have been associated with the potential spread of food-borne diseases (Graczyk *et al.*, 2005). The higher Diptera includes the family Muscidae whose most known members are commonly called houseflies (*Musca domestica* L) (Nihei and De Carvalho, 2007; Gupta *et al.*, 2012).

2.2 CHARACTERISTICS AND MORPHOLOGICAL IDENTIFICATION

Houseflies are non-metallic medium-sized (6–9 mm long mm in length) and vary in colour from light to dark grey with some darker markings (Service, 2012). These insects are characterized by the presence of (i) four moderately broad black longitudinal stripes on the dorsal surface of the thorax (ii) four visible greyish abdominal segments, which are usually partially covered by the wings, (iii) the antennae, which are not easily seen, are concealed in despairs on the front of the face, and (iv) the spongy-sucking mouthparts which are specially adapted for sucking up fluid or semifluid foods and are partially withdrawn into the head capsule when not in use (Service, 2012). The wings of the house fly have vein 4 bending up sharply to join a thick vein along the front edge of the wing close to vein 3 (Figure 2.1). This important identification character is of assistance to distinguish *Musca* species from other rather similar flies. The three pairs of legs end in paired claws and pulvilli, which are supplied with glandular hairs. These sticky hairs enable the housefly to adhere to smooth surfaces including glass windows and to pick up pathogens (Service, 2012).

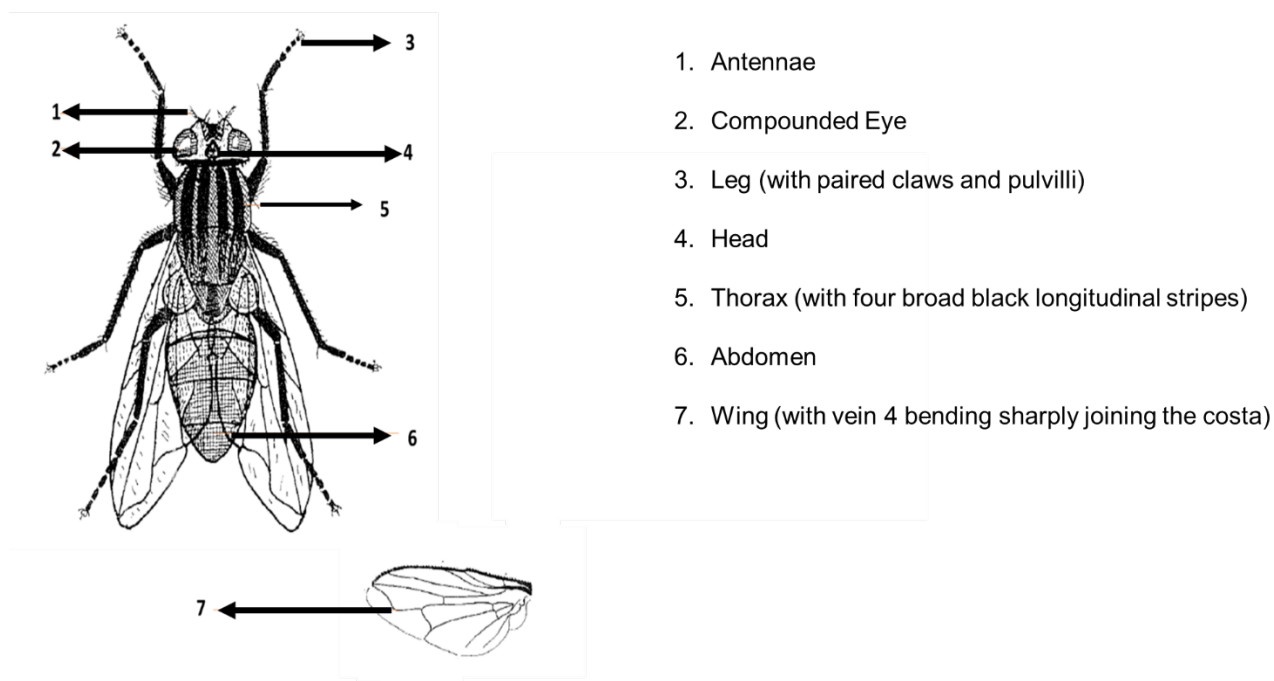


Figure 2.1 Adult housefly morphological illustration and wing indication (Service, 2012).

2.3 LIFE CYCLE OF A HOUSEFLY

Houseflies have more broods of offspring per year and go through 10 to 12 generations annually in temperate regions with populations peaking in summer (Graczyk *et al.*, 2005). Winter usually ends the breeding cycle; however, flies from indoors can develop several generations during the winter seasons (Graczyk *et al.*, 2005). Cattle barns and other animal-associated locations that are warm enough and offer sufficient development sites and food to support the houseflies' life cycle, for example, are sites where houseflies can breed throughout the winter (Graczyk *et al.*, 2005).

Houseflies have a complete metamorphosis with egg, larval, pupal and adult stages; (Figure 2.2). Adult female houseflies can live 15 to 25 days and lay eggs, which are either deposited together or in five to six batches of 75 to 150 batches (Graczyk *et al.*, 2005; Service, 2012; Sanchez-Arroyo and Capinera, 2014). This insect lay eggs preferably on decomposing materials including animal manure, poultry dung, urine-contaminated bedding, carcasses, decomposing organic materials from rubbish dumps, household garbage and waste foods from kitchens (Service, 2012). The eggs hatch into larvae (maggots) and develop into three instars in about 7 to 9 days. This maggot consists of a

small head with an 11-segmented cylindrical body. The rapidity of larval development depends on the abundance of food and temperature. Third-instar larvae often move to the drier ground before pupation. Pupation begins with the larval skin contracting, hardening and turning dark brown, resulting in a barrel-shaped structure about 6 mm long, called a pupa which is segmented. The adult housefly emerges from the puparial case by pushing off the anterior end and crawling out, and after a short period, it flies away.

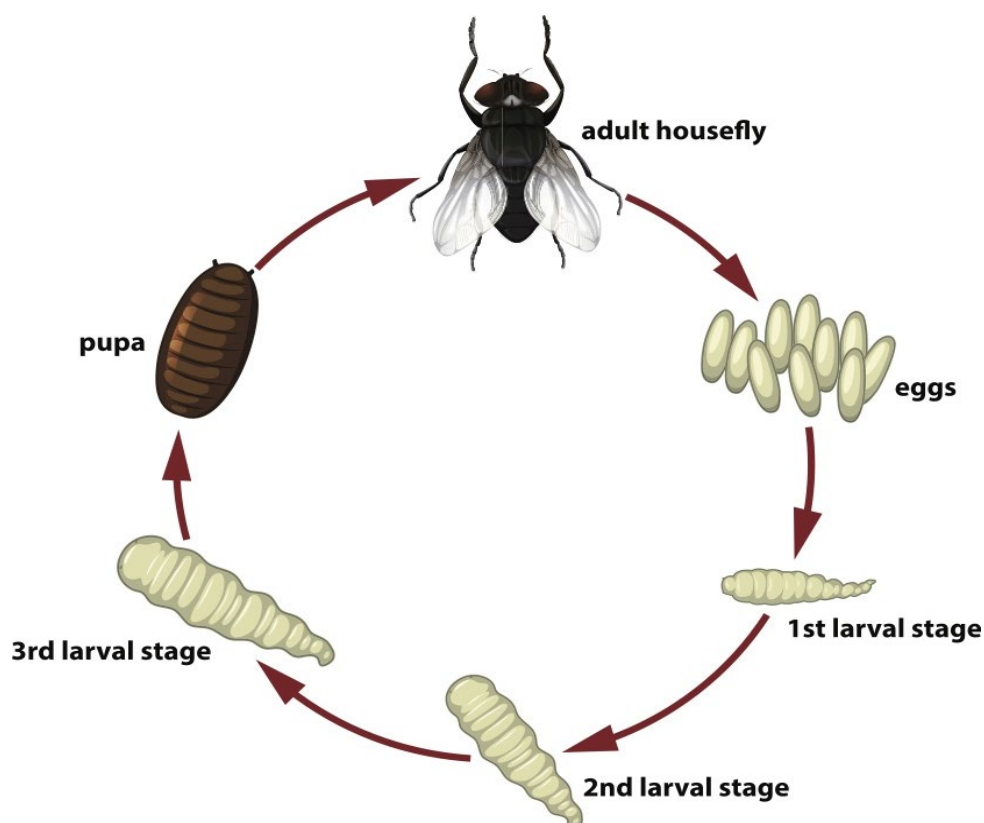


Figure 2.2 Complete life cycle of housefly including larvae, pupae and adult stages (<https://www.ripleys.com/weird-news/houseflies/>).

2.4 ASSOCIATION OF HOUSEFLIES WITH HUMAN HABITAT

Non-biting members of the genera *Musca* consist of many species and only a few have become closely associated with human dwelling places hence the name houseflies (Hamed *et al.*, 2007). Houseflies have been implicated in the transmission of solemn diseases including anthrax, ophthalmia, typhoid fever, tuberculosis, cholera, and infantile diarrhea (Gupta *et al.*, 2012). Houseflies are abundant in urban and rural areas where mostly unhygienic conditions prevail and are usually scarce when hygienic conditions are enforced (Graczyk *et al.*, 2005).

Houseflies are known to disperse over a wide area often with no superficial patterns regarding wind direction, food, water proximity, or suitable mating and larval development sites (Echeverria *et al.*, 1983; Barreiro *et al.*, 2013). They are abundant in environments that include open marketplaces, households, carnivals, restaurants, garbage junkyards, animal production, and restricted animal feeding operations (Echeverria *et al.*, 1983; Barreiro *et al.*, 2013).

2.5 HOUSEFLIES AS VECTORS OF PATHOGENS AND DISEASE

Associations of houseflies with humans and attraction to both filth and human food enhance its role as a vector of disease-causing pathogens (Graczyk *et al.*, 2005). Houseflies manifest several degrees of synanthropic, and some are significant from a medical and veterinary point of view (Hamed *et al.*, 2007; Gupta *et al.*, 2012). Houseflies are distinguished for their annoyance to humans as well as ectoparasites of animals and disease vectors (Zhu *et al.*, 2016).

Houseflies have been demonstrated to transmit pathogens from their sponging-sucking mouthparts, through vomitus, or on body and leg hairs, or by adhesive pads on the feet called pulvilli and through the intestinal tract (De Jesus *et al.*, 2004). This insect harbours bacteria in both immature and mature life stages (Zurek *et al.*, 2000), and is widely considered a significant source of diverse and active microbial communities (Wei *et al.*, 2013). The source of these active microbial communities includes reservoirs such as manure, human refuse, open privies, soiled animal bedding, litter and waste around food and vegetables processing plants since they reproduce and develop in such places (Gupta *et al.*, 2012; Barreiro *et al.*, 2013). Houseflies greatly amplify the risk of human exposure to foodborne pathogens and other pathogenic and multidrug-resistant bacteria. This is due to houseflies' (i) indiscriminate movements, (ii) ability to fly long distances, (iii) attraction to both decaying organic materials (iv) places where food is stored or prepared (Gupta *et al.*, 2012; Barreiro *et al.*, 2013).

Houseflies contribute to the mechanical transmission of food-borne pathogens and other serious disease causing organisms that can negatively affect humans and animals, this includes anthrax, ophthalmia, typhoid fever, tuberculosis, certain skin infections, polio,

cholera, and infantile diarrhoea among others (Parvez *et al.*, 2016; Nazari *et al.*, 2017). Transmission takes place mostly when the houseflies are in contact with humans and their food (Bahrndorff *et al.*, 2017). Food-borne diarrheal disease outbreaks have been reported in urban and rural areas, and they are said to be related to a seasonal increase in the abundance of houseflies (Graczyk *et al.*, 2005). As such, enforced fly control measures are correlated with the reduction in the number of cases of such a disease (Graczyk *et al.*, 2005).

2.6 VECTOR, PATHOGEN AND HOST RELATIONSHIP

Houseflies develop as larvae in a wide range of decayed, fermenting, or rotting organic material that is rich in microbial communities and give supports to the proper development of the larvae (Zurek *et al.*, 2000). These may include human and animal faeces, rotting vegetation and garbage (Graczyk *et al.*, 2005). The larvae are in close contact and consume these microbes, therefore, consequently harbouring them throughout the different life stages (Rochon *et al.*, 2005). Thus, all trophic levels of housefly including larvae, pupae and adults are commonly contaminated with various microorganisms (Gupta *et al.*, 2012).

The gut microbiota represents all aspects of microbial relationships, from pathogenic to obligate mutualism (Dillon and Dillon, 2004). Therefore, the interactions amongst housefly's host and microbes can be described in several ways including, mutualistic, commensal and parasitic (Wei *et al.*, 2013). Bacteria play a significant role in the successful development of housefly larvae. Bahrndorff *et al.* (2017) demonstrated that house fly larvae fail to grow in an axenic environment. The microbiota of houseflies can contribute to various aspects of host physiology including nutritional supplementation, tolerance to environmental perturbations, providing colonization resistance against pathogenic organisms, and priming the development and maturation of the fly's immune system (Bahrndorff *et al.*, 2017). Variation in the microbiota between individual houseflies explains differences in the phenotype, such as vector competency (Bahrndorff *et al.*, 2017).

2.7 BACTERIAL COMMUNITIES

Bacteria are social organisms that interact extensively within and between species all while responding to external stimuli from the environment they inhabit. Indeed, the ability to perceive neighbouring cells and the environment is often reflected in the content of bacterial genomes (Stubbendieck *et al.*, 2016). Interactions between hosts and microorganisms are increasingly recognized as a ubiquitous principle in nature (Junqueira *et al.*, 2017). Thus, bacterial communities vary in their species composition, niches occupied, and influence on different environments (Stubbendieck *et al.*, 2016; Zhou *et al.*, 2015). Houseflies have shown to harbour pathogenic bacteria that include *Salmonella* spp., *Proteus* spp., *Shigella* spp., *Chlamydia* spp., *Campylobacter jejuni*, *Klebsiella* spp., *Escherichia coli* O157:H7, *Yersinia pseudotuberculosis*, *Helicobacter pylori* and *Pseudomonas* spp., *Vibrio cholerae*, Enterobacteriaceae pathogens as well as *Staphylococcus aureus* (Gupta *et al.*, 2012; Wei *et al.*, 2013; Bahrndorff *et al.*, 2017). According to Park *et al.*, (2019), houseflies harbour a very diverse microbiota, which is influenced by habitat and geographical origin. Since the different bacterial species found on houseflies depend on the location where the insects are captured (Cervelin *et al.*, 2018).

2.8 APPROACHES TO DETECTION OF BACTERIAL COMMUNITIES

2.8.1 Culture-dependent approach

The culture-dependent approach simply provides a good indication of ecosystem complexity. However, the approach does not necessarily provide comprehensive information on the composition of microbial communities. Hence this approach is limited to cultivable organisms (Gupta *et al.*, 2012). This type of approach is only useful in understanding the physiological, metabolic and biochemical potential of isolated microorganisms (Gupta *et al.*, 2012). Culture-dependent technique was mostly used by many to recover housefly-associated microbiota, and mainly focusing on specific pathogenic bacterial strains (Forster *et al.*, 2007; Graczyk *et al.*, 2001).

During culture-dependent assays, one employs culturing alone or culture method followed by PCR focusing on limited or single species (Gupta *et al.*, 2012). The outcomes

of this technique were found to be limited since less-than one percent (>1%) of bacterial strains in the environment can be cultivated. This simply means ~99% of all bacteria available in the environment are uncultivable (Rappe and Giovannoni, 2003; Wei *et al.*, 2014).

New technologies such as MALDI-TOF-IMS (Matrix-assisted laser desorption/ionization [MALDI] time-of-flight [TOF] imaging mass spectrometry [IMS]) have enabled examination of bacterial communities at small three-dimensional scales. However, studies of natural communities continue to be challenged by the difficulty in culturing numerous bacterial communities from the environmental samples in the laboratory settings (Stubbendieck *et al.*, 2016).

2.8.2 Culture-independent approach

There is a need to investigate the entire bacterial population, including strains that cannot be cultivated under laboratory conditions. This will better the understanding of host-microbial interactions and assess the persistence of pathogenic or antibiotic-resistant bacteria in a bacterial community (Wei *et al.*, 2013). Metagenomics has become a powerful approach in determining the composition and connectivity of bacteria in a community (Stubbendieck *et al.*, 2016).

Molecular-based techniques revealed that more than 99% of microorganisms existing in nature could not be isolated and maintained in pure culture (Palavesam *et al.*, 2012). Culture-independent methods allow an opportunity to identify both cultivable and non-cultivable fractions of microbes present in the given sample. These are molecular-based methods that require DNA extractions, polymerase chain reaction (PCR), and cloning conditions to be standardized to prevent the introduction of bias due to primer selection or low representation of genera or species in the microbial community (Gupta *et al.*, 2012).

Recent advancements in DNA sequencing technology and bioinformatics offer a beneficial and efficient tool to analyse bacterial communities of insects. This eliminates the need for intensive culture-based techniques and allows for the identification of bacterial species that are uncultivable (Palavesam *et al.*, 2012). Extensive detailed surveys using culture-independent method including pyrosequencing have been carried

out to reveal fly- associated bacterial communities (Wong *et al.*, 2011; Zheng *et al.*, 2013; Kautz *et al.*, 2013). Bahrndorff *et al.*, (2017) indicated that the use of culture-independent amplicon sequencing of the *16S rRNA* gene to characterize the bacterial communities and richness associated with individual houseflies allows greater resolution addressing their microbiota. The use of both approaches along with taxonomy and phylogenetic techniques allows for better and more thorough characterization of microbial diversity in insects (Gupta *et al.*, 2012).

Investigation of the bacterial community from *16S rRNA* gene sequencing experiments includes linking the reads to a reference database (Lu and Salzberg, 2020). Thus, the choice of the *16S* database is of great importance and significance to avoid challenges that may affect post-analysis and interpretation of the community composition (Park and Won, 2018). There are few standard *16S rRNA* databases available for microbial identification, each with different content (Park and Won, 2018; Lu and Salzberg, 2020). Public databases providing taxonomy include Greengenes (<http://greengenes.secondgenome.com>), SILVA (<https://www.arb-silva.de>), Ribosomal Database Project (RDP; <http://rdp.cme.msu.edu/>) and EzBioCloud (<https://www.ezbiocloud.net>); these databases are currently being used extensively in amplicon sequencing analysis (Cole *et al.*, 2014; Park and Won, 2018; Lu and Salzberg, 2020). The tool most widely used for *16S rRNA* analysis and classification to date is the Quantitative Insights into Microbial Ecology (QIIME) software package (<http://qiime.org/index.html>), which compares sequencing reads against a *16S rRNA* reference database (Caporaso *et al.*, 2010; Lu and Salzberg, 2020). Thus, Greengenes is the most popular and widely used database, since it is the default database in the QIIME pipeline (Park and Won, 2018).

2.9 ANTIBIOTIC RESISTANT BACTERIA

Antibiotics are antimicrobial agents naturally produced by bacteria or fungi to inhibit the growth of other microorganisms. They are used in medicine to treat infections caused by microbes and they are widely used as growth supplements in livestock (Ventola, 2015). Though antibiotics are essential to both human and animal health, recently, some bacteria have demonstrated resistance to various antimicrobial agents resulting in a

phenomenon known as antimicrobial resistance (Davari *et al.*, 2010; Solà-Ginés *et al.*, 2015).

Antimicrobial resistance is problematic and of major concern in the public health system because it threatens the effective prevention and treatment of an ever-increasing range of infections caused by bacteria (Wei *et al.*, 2013). Antibiotic resistance is defined as a phenotypic property: the ability of a bacterial cell to survive and grow in the presence of an antibiotic concentration that is inhibitory to susceptible cells (Luby *et al.*, 2016). Bacterial cells can develop resistance to antibiotics by one of the following routes: (i) spontaneous mutation and (ii) horizontal gene transfer.

Spontaneous genes refer to when bacterial cells confer resistance to an antibiotic by modifying the antibiotic's target or its expression level, or by up-regulating resistance genes, such as those encoding efflux pumps (Baym *et al.*, 2016). The housefly digestive tract provides a suitable environment for horizontal gene transfers among bacterial cells leading to the spread of resistant and virulent bacterial genes (Liu *et al.*, 2013). Therefore, the bacterial cell can acquire resistance genes through horizontal gene transfer; the genes will therefore encode specialized antibiotic degradation enzymes, efflux pumps, target protection proteins, or bypass pathways (Baym *et al.*, 2016). Horizontal gene transfer occurs by sharing of genes between bacterial cells via different mechanisms where mobile genetic elements, including bacteriophages, plasmids, integrons and transposons mediate the transfer (Gyles and Boerlin, 2014).

The increasing frequency of antibiotic resistance has been reported from all over the world. The cost of health care for patients with resistant infections is higher than care for patients with non-resistant infections due to the longer duration of illness, the requirement for additional tests, and the use of more expensive drugs (Wei *et al.*, 2013). Hence housefly-mediated dissemination of antibiotic-resistant bacteria increasingly draws attention. Therefore, an attempt to determine the frequency of resistance and susceptible bacteria isolated from houseflies is of importance (Davari *et al.*, 2010). Houseflies collected in hospitals and slaughterhouses may be involved in the spread of drug-resistant bacteria and may increase the potential for human exposure to drug-resistant bacteria (Davari *et al.*, 2010).

The development of antibiotic resistance among clinical bacterial isolates and commensal bacteria of people and animals, as well as bacterial cells in other habitats, raises a concern that flies may be a vector competent not only for specific pathogens but also for non-pathogenic bacteria carrying antibiotic-resistant and virulent genes (Barreiro *et al.*, 2013). Recently, antibiotic-resistant bacteria, especially multiple antibiotic-resistant strains of pathogenic bacteria, have also been found in flies in hospital environments or near animal production sites (Wei *et al.*, 2013).

The close association of houseflies with bacteria and their role in the transmission of pathogens makes it an ideal model organism to study the importance and variation in the microbiota of vector species (Bahrndorff *et al.*, 2017). However, few studies have addressed the variation in the microbiota of filth flies under natural conditions although such variation is likely to affect the phenotype of the housefly (Bahrndorff *et al.*, 2017). Microbiota analysis will highlight the presence of symbionts harboured by houseflies as well as microbiota of public health significance.

2.10 ANTIBIOTIC RESISTANCE GENES AND MECHANISM

Antibiotic resistance genes encode the ability for bacterial cells to grow in the presence of antibiotics. This is accomplished by encoding various functions including efflux pumping, degrading the antibiotic, or modifying the target (Luby *et al.*, 2016). Antibiotic resistance bacterial cell displays one of the following three fundamental phenotypes including (i) susceptibility, (ii) intrinsic resistance, (iii) or acquired resistance (Harbottle *et al.*, 2006). Intrinsic resistance can be described as a natural phenomenon that is displayed by all members of a species and is a function of the physiological or biochemical makeup of that species. Acquired resistance can result from the mutation of regulatory or structural genes, the acquisition of foreign resistance genes, or a combination of these two mechanisms (Harbottle *et al.*, 2006).

Numerous genes can be responsible for antibiotic resistance in bacterial cells, these genes are also able to be transferred between bacterial cells (Nazari *et al.*, 2017). However, there is a limited number of mechanisms by which these resistance traits are acquired (Harbottle *et al.*, 2006). Genes encoding antibiotic resistance determinants may be located on the chromosome, where they are inherited by daughter cells, or they might

be horizontally transmitted on mobile DNA elements, such as plasmids transposons and integrons (Harbottle *et al.*, 2006). The transmissible plasmids also carry virulence determinants and the acquisition of resistance and virulent traits may provide a survival benefit to the microorganism (Beceiro *et al.*, 2013). The ability of bacterial populations to adapt to toxic environments and their ability to exchange DNA indicates that antibiotic resistance is a biological phenomenon that will continue to be a chronic public health problem (Harbottle *et al.*, 2006). Resistance phenotypes caused by point mutations often alter the binding targets of antimicrobials, resulting in lowered binding affinity (Harbottle *et al.*, 2006). In addition to mutation, resistance also results from degradation or alteration of the antimicrobial, reduced uptake of an antimicrobial, and active efflux of an antimicrobial out of the cell (Harbottle *et al.*, 2006).

2.11 DETECTION OF ANTIBIOTIC RESISTANT PATHOGENS

Detection of an antibiotic resistance gene indicates potential resistance due to the following reasons: (i) the gene may not be expressed, (ii) the gene may have mutated to a non-functional form, (iii) the gene may be present in a dead cell or as extracellular DNA, and or (iv) the gene may be incomplete because most methods detect portions of the antibiotic resistance genes and not the entire reading frame (Luby *et al.*, 2016).

2.11.1 Phenotypic detection methods

Antibiotic resistance is a selectable phenotype and can be detected using growth inhibition assays. Phenotypic disc diffusion and minimum inhibitory concentration (MIC) determinations have been the gold standard for antimicrobial susceptibility testing (Zankari *et al.*, 2012). These methods have the great advantage of determining the ‘true’ *in vitro* relationship between the antimicrobial agent and the strain tested and will detect any new emerging resistance mechanisms. Phenotypic results are obtained traditionally by antimicrobial sensitivity testing and are vital part of clinical investigations related to bacterial infections (Anjum *et al.*, 2017).

2.11.2 Molecular detection of antibiotic resistance

Identification of antibiotic resistance genes is important for understanding the underlying mechanisms and the epidemiology of antibiotic resistance. Molecular methods are often

used in addition to phenotypic methods but are set to replace them in many laboratories due to the greater speed and accuracy they provide in detecting the underlying genetic mechanisms for antibiotic resistance (Aarts *et al.*, 2001; Anjum *et al.*, 2017). Some of the common molecular methods currently used for the detection of antibiotic resistance genes include (i) polymerase chain reaction (PCR), (ii) DNA microarray, (iii) whole-genome sequencing and metagenomics, (iv) and matrix-assisted laser desorption ionization-time of flight mass spectrometry (Schwartz *et al.*, 2003; Czelkalski *et al.*, 2014; Anjum *et al.*, 2017). However, these culture-independent approaches only target well-studied pathogens or resistance-causing genes and cannot easily be used for broad-spectrum screening. Thus, molecular characterization methods are often used as an indirect method of support when phenotypic data is insufficient to control resistant bacteria (Aarts *et al.*, 2001; Anjum *et al.*, 2017).

2.12 CONTROL MEASURES OF HOUSE FLY POPULATIONS

Housefly populations can be controlled by insecticides or by physical means such as sticky tapes, fly swats and electrocuting grids. However, improved environmental, sanitation and hygiene are vital in effectively controlling houseflies (World Health Organization, 2011). According to WHO (2011), environmental, sanitation and hygiene improvement can be achieved by (i) reducing or eliminating of housefly breeding sites, (ii) reducing source that attracts houseflies from other areas, (iii) preventing contact between houseflies and disease-causing pathogens and (iv) protecting food, eating utensils and people from contact with houseflies

In areas where livestock farming is practiced, controlling flies is more challenging as these areas are ideal habitats in terms of breeding and feeding for the flies. Hence, rural areas near the farmland are the most chosen for housefly sampling, this was, however, not the case in the current study (Forster *et al.*, 2009; Barreiro *et al.*, 2013). Houseflies are frequently found resting on animals, animal manure and decaying food, and they can easily pick up microorganisms available on those sites and transport them to a private house or kitchen, thus becoming high-risk vectors (Barreiro *et al.*, 2013). Hence the focus of the current study was on houseflies collected in hospices. The hospice institutions are intended to increase the quality of life for terminally ill patients (Datta and Juthani-Mehta,

2017). According to Furuno *et al.*, 2015 hospice patients are at high risk of infections owing to decreased host resistance, multiple comorbid illnesses and recurrent health exposures.

The current study sought to document the occurrence and characterize houseflies sampled from the hospices in the Bloemfontein and Johannesburg cities of South Africa. Furthermore, the study identified bacterial communities harboured by these houseflies using metabarcoding on the MiSeq next-generation sequencing (NGS) platform and also determine the antibiotic resistance and virulence genes in microbial communities associated with houseflies by polymerase chain reaction assays.

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CHAPTER 3

BACTERIAL PATHOGENS IDENTIFIED FROM HOUSEFLIES IN DIFFERENT HUMAN AND ANIMAL SETTINGS: A SYSTEMATIC REVIEW AND META-ANALYSIS

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3.1 INTRODUCTION

Houseflies exist as major pests of humans, poultry and livestock surroundings and facilities where they transmit vector-borne infections globally (Ahmed *et al.*, 2013; Iqbal *et al.*, 2014; Akter *et al.*, 2020). Based on World Health Organization (WHO) records, the major vector-borne infections account for almost 17% of communicable diseases that occur per annum worldwide, with high incidence in tropical and subtropical regions (World Health Organization, 2017).

Houseflies are common and occur in high abundance in close association with human activities. These insects are attracted to human and animal waste for feeding and reproduction (Awache and Forouk, 2016). Human (i.e., hospitals, food markets and restaurants) and animal environments (i.e., farms, feedlots, and slaughterhouses) are the major points of focus and sampled areas for houseflies. Owing to their diverse habitat preference, indiscriminate movement, the ability to fly long distances and attraction to decaying organic materials, houseflies greatly amplify the risk of human exposure to a variety of pathogens including bacteria (Barreiro *et al.*, 2013; Gupta *et al.*, 2012).

The reproduction habits and feeding mechanisms of houseflies involve microbe-rich substrates (Park *et al.*, 2019). Thus, houseflies harbour bacteria in both immature and mature life stages (Zurek *et al.*, 2000), and are widely considered a significant source of bacterial diversity (Wei *et al.*, 2013). The bacterial species found to colonize houseflies include *Salmonella* spp., *Yersinia enterocolitica*, *Edwardsiella tarda*, *Shigella sonnei*,

Escherichia coli, *Klebsiella* spp., *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Enterococcus faecalis* (Rahuma *et al.*, 2005), to name a few. The characteristics of bacterial genera carried by houseflies are dependent on the sampled area (Khamesipour *et al.*, 2018). Thus, houseflies can transport a diverse and active microbial community from different reservoirs (Gupta *et al.*, 2012; Barreiro *et al.*, 2013). Therefore, these pathogens may cause serious infections and diseases to susceptible humans and animals (Malik *et al.*, 2007; Ahmed *et al.*, 2013).

The risk of houseflies disseminating antibiotic-resistant bacterial strains from hospital settings, livestock and poultry farms to public residents is of great concern (Zhang *et al.*, 2018). According to Zhang *et al.*, (2018), antibiotic-resistant bacteria including bacterial species that are multi-drug resistant, commonly colonize houseflies sampled from these areas with extensive usage of antibiotics. An increasing frequency of antibiotic resistance has been reported from all over the world (Davari *et al.*, 2010). Thus, the emergence of bacteria that is resistant to critically important antimicrobials is of major concern in human and veterinary medicine (Solà-Ginés *et al.*, 2015).

Houseflies colonized with these bacterial species may also be associated with the dissemination of antibiotic-resistant genes or exposure to virulent bacterial strains within the same environment (Bouamama *et al.*, 2010; Zhang *et al.*, 2018). Transmission takes place mostly when the houseflies come in contact with animals, people and their food (World Health Organization, 1997). Thus, houseflies contribute to the mechanical transmission of foodborne pathogens and other serious diseases that can affect humans and animals. These diseases include anthrax, ophthalmia, typhoid fever, tuberculosis, cholera and infantile diarrhoea (World Health Organization, 1997; Khamesipour *et al.*, 2018).

The current systematic review and meta-analysis were carried out to determine the pathogenic bacteria isolated from houseflies by various studies conducted around the world, and how frequently these studies report on the antibiotic susceptibility profiles and resistant genes in common bacterial pathogens. This was performed to generate evidence and knowledge necessary to help formulate preventative measures against infectious bacteria harboured by houseflies.

3.2 MATERIALS AND METHOD

3.2.1 Systematic search strategy and selection criteria of the journal articles

This review was done following the recommended methodology as outlined by the preferred reporting items for systematic reviews (PRISMA) as shown in Figure 1 (Moher et al., 2015). The literature search was performed to identify journal articles published from 1980 to 2020 reporting on houseflies or *Musca domestica* and bacteria. The phrase “*Musca domestica*” was searched following the combination of free text and thesaurus terms in different variations: houseflies, bacterial pathogens. These keywords were used to retrieve studies from electronic databases including PubMed (<https://pubmed.ncbi.nlm.nih.gov/>, from 01/09/2019 to 08/06/2020); Science Direct (<https://www.sciencedirect.com/> from 06/09/2019 to 08/06/2020); and Google scholar (<https://scholar.google.com/> from 11/09/2019 to 08/06/2020) with the last search done on the 8th of June 2020. The systematic search was accomplished following the combination of free text and wordlist terms in diverse distinctions: “Houseflies” AND/OR, “*Musca domestica*”, “Bacterial pathogens” AND/OR “Bacterial isolation”. Thereafter, titles and abstracts were scanned and potential journal articles were reviewed and downloaded. The reference list from the potential searched published journal articles was also screened for journal articles relating to this review.

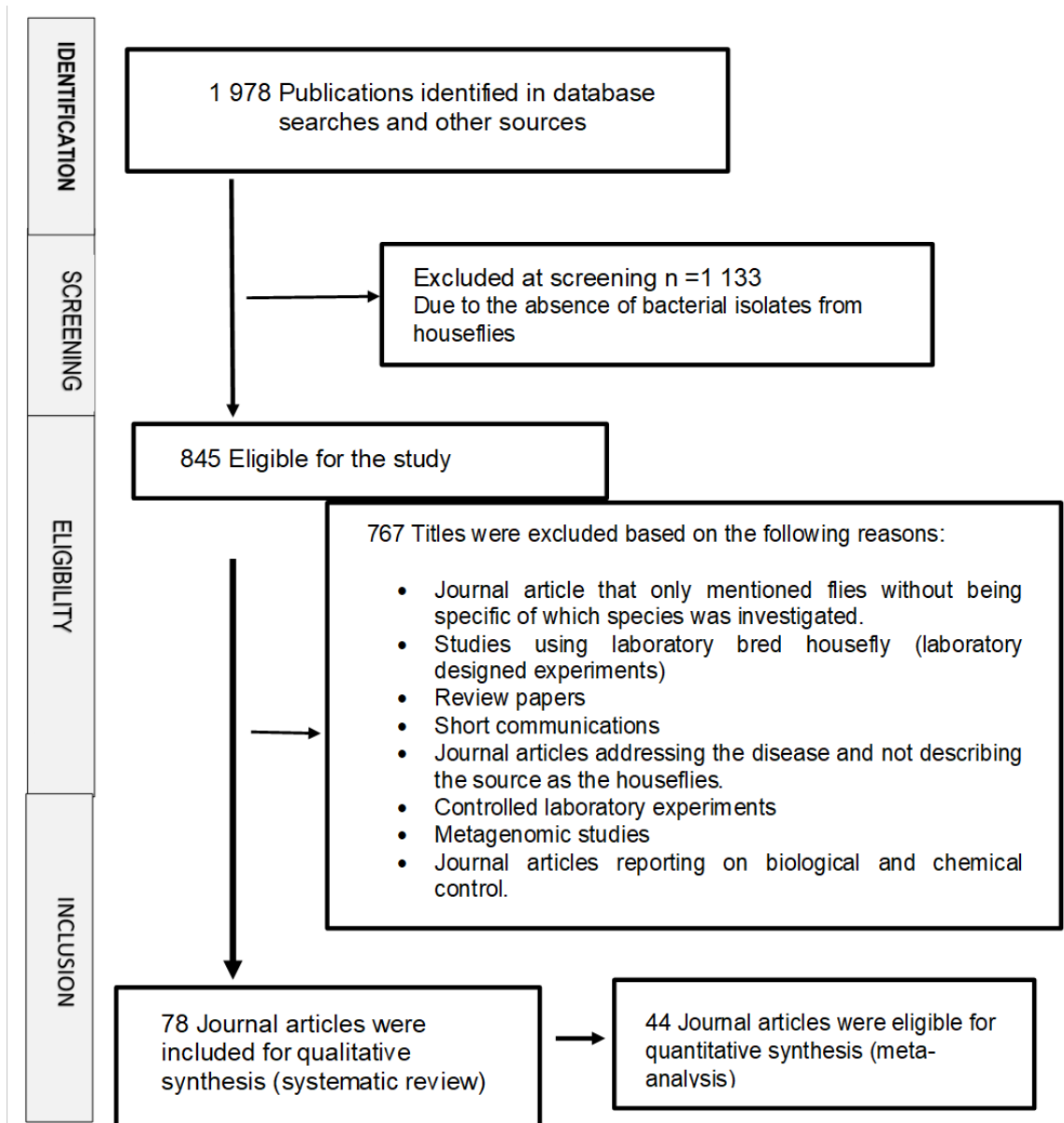


Figure 3.1 The selection process of eligible studies following PRISMA guidelines

3.2.2 Inclusion and exclusion criteria for systematic review

The search was limited to only the English language and was conducted from 01 September 2019 to 08 June 2020. The abstracts were assessed and the scientific papers that met a preceding inclusion criterion were selected. Preliminary screening of titles and abstracts was carried out for eligibility and relevance to this systematic review and meta-analysis according to the inclusion and exclusion criteria. The published journal articles selected in this study were read for data extraction and screened further by a detailed review of the full text. The articles had to fulfil the following criteria to be included: (i)

original research articles from around the world (ii) the availability of the full texts (iii) journal articles published in English language (iv) conducted between 01 September 2019 to 08 June 2020; (v) study design was cross-sectional/prevalence study; (vi) the laboratory examination methods used was clearly stated; (vii) the geographical location of the study was clearly stated; (viii) the species of *Musca domestica* was clearly stated; (ix) the number of *M. domestica* with bacterial positive cases and sample size were provided; (x) the study screened for all bacterial species. Articles were excluded if they were published only in abstract form, or if they were editorials, letters, comments, or reviews. Furthermore, the excluded articles were those reporting pathogens isolated from flies in general without specifying the fly species, studies using laboratory-reared *M. domestica* (laboratory designed experiments), journal articles addressing the disease and not describing the source as *M. domestica*, controlled laboratory experiments and metagenomic studies (Figure 3.1). No attempt was made to contact the authors of the original manuscripts for any additional information or retrieval of unpublished studies. The data was retrieved by one investigator and then confirmed by another investigator.

3.2.3 Data extraction and Meta-analysis

Key characteristics of each included article were summarized in a spreadsheet (Microsoft Excel ®), with the following variables: author name, country; sites of sample collection, diagnostic technique and bacterial isolates. Several bacteria species were reported from all the included studies. For the sampling locations, closely related sampled places were grouped together. Only journal articles which were definite with the species of bacteria were included for meta-analysis. Five selected pathogenic bacteria namely, *Escherichia coli*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* were focused on. The prevalence for the subgroups were calculated as follows; the number of bacterial species divided by the sample size with the results expressed in percentages (Table 3.2). However, some studies used duplicates or replicate from the same fly homogenates. Thus, the number of positive bacterial species was recorded at 100% prevalence if the number recorded exceeds the sample size.

3.2.4 Statistical analysis

Meta-analysis was carried out using Comprehensive Meta-Analysis Version 3.0. Between the studies, heterogeneity was assessed using Higgin's I^2 (Inverse variance) and Cochran's Q method. I^2 values of 25%, 50%, and 75% were considered low, moderate and high heterogeneity, respectively (Higgins and Thompson, 2002). Subgroup analysis was also conducted by the location of the study, study sites and diagnosis method used. The effect size and their corresponding confidence interval (95% CI) for each subgroup were calculated and expressed on the forest plots. A funnel plot was generated to evaluate the potential for asymmetry or publication bias present.

3.3 RESULTS

3.3.1 Study selection

Following a systematic approach and bibliographic search, 1 978 references were retrieved from the initial search strategy (Figure 3.1). Citation searches were based on published journal articles. Overall, 1133 studies were excluded following the initial screening based on titles or abstracts and duplicate journal articles were also uninvolved. The remaining 845 journal articles were further screened for eligibility and 767 studies were excluded for various reasons stipulated in Figure 3.1.

3.3.2 Study characteristics of eligible studies

Characteristics of the eligible studies include the number of houseflies collected, bacterial species isolated, identification method, country and sampled sites as presented in Table 3.1. All the journal articles were published between the years 1980 and 2020. A handful of studies have been conducted globally that reports on bacterial pathogens isolated from houseflies. Studies included in this review were from four different continents namely, Africa, the Americas, Asia and Europe (Figure 3.2). Asia (47.4%) was observed to have the highest number of studies followed by the Americas (23.1%) and Africa (20.5%), and Europe (8.9%) being the least. The sample size from eligible studies ranged from 10 houseflies to 3440 individual houseflies' specimens. Samples were collected from diverse

sites including hospitals, water treatment facilities, slaughterhouses, garbage dumps, farms and residential areas. Thus, 38 (48.7%) studies were sampled from more than one site, whilst 39 (50%) of the studies sampled only from one specific site and one (1.3%) study failed to report on their sampled site (Table 3.1).

The techniques employed by the eligible studies to isolate and identify bacterial species included culture-dependent (microbiological culture) and culture-independent (polymerase chain reaction (PCR)) respectively. However, other studies that made use of both techniques simultaneously were also included in the analysis. At least 56.4% (44/78) of the studies used the culture method for the identification of pathogens, 30.7% (24/78) used the combination of both culture, and PCR diagnostic technique for the identification and only 7.7% (6/78) used the PCR method for the identification. However, one study failed to mention their diagnostic technique. Of 39/78 (50%) studies, reported on antibiotic susceptibility profile for different bacterial genera and only 13/78 (16.7%) studies reported on antibiotic-resistant genes. The bacterial species reported in this review are recognized pathogens including *Escherichia coli*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. Amplification of some common resistance genes for colistin, sulphonamide, tetracycline, beta-lactam, penicillin, quinolones and integrons were observed in this study (Table 3.1).

Table 3.1 List and characteristics of eligible studies included in the systematic review

References	Number of samples	Bacterial species isolated	Diagnostic method	Country	Sampled sites	Antibiotic susceptibility profile	Resistant gene
1. Rosef <i>et al.</i> , 1983	608	<i>Campylobacter fetus</i> subsp. <i>jejuni</i>	Culture	Norway	Cattle barn Turkey farm Piggery farm Chicken farm	None	None
2. Khin-Nwe <i>et al.</i> , 1989	186	<i>Escherichia coli</i> <i>Vibrio cholerae</i> <i>Salmonella</i> spp. <i>Shigella</i> spp.	Culture	Myanmar	Animal pens Kitchen Bazaar Latrine Refusal dumps Food stalls	None	None
3. Fotedar <i>et al.</i> , 1992a	240	<i>Klebsiella</i> spp. <i>Klebsiella pneumoniae</i> <i>Klebsiella oxytoca</i> <i>Klebsiella ozanae</i> <i>Klebsiella rhinoscleromatis</i>	Culture	India	Surgery ward- Hospital Residential areas	Amikacin, Streptomycin Gentamicin Tetracycline Erythromycin Chloromycetin Sisomicin Cephalexin Ampicillin	None
4. Fotedar <i>et al.</i> , 1992b	113	<i>Klebsiella</i> spp. <i>Pseudomonas aeruginosa</i> <i>Escherichia coli</i> <i>Enterobacter</i> spp.	Culture	India	Hospital Residential area	None	None

		<i>Proteus spp.</i> <i>Acinetobacter spp.</i> <i>Staphylococcus aureus*</i> <i>Staphylococcus epidermidis</i> <i>Viridans streptococci</i> <i>Enterococcus faecalis</i> <i>Other Streptococci</i> <i>Micrococci</i> <i>Bacillus spp</i>					
5. Khalil <i>et al.</i> , 1994	300	<i>Campylobacter spp.</i> <i>Escherichia coli</i> <i>Shigella spp.</i> <i>Salmonella spp.</i>	Culture	Pakistan	Restaurants Roadside barbecue shops Sweet candy shops Butchers Hawkers Street vendors	None	None
6. Buma <i>et al.</i> , 1999	None	<i>Escherichia coli</i> O157: H7,	Culture PCR	Japan	Dairy farm field	Ampicillin Chloramphenicol Erythromycin Tetracycline Kanamycin Lincomycin Cefazolin Colistin	None

						Ciprofloxacin Sulfamethoxazole /trimethoprim	
7. Iwasa <i>et al.</i> , 1999	310	<i>Escherichia coli</i> O157:H7	Culture PCR	Japan	Cattle farm Pig farm Poultry farm	Fosfomycin Tetracycline Minocycline Streptomycin Ampicillin Penicillin G Chloramphenicol Kanamycin Nalidixic-acid Noroxacin Cefdinir Trimethoprim	None
8. Moriya <i>et al.</i> , 1999	10	Verotoxin-producing <i>Escherichia coli</i> O157:H7	Culture	Japan	Residential area	None	
9. Olsen and Hammack.,2000	15	<i>Salmonella</i> spp.	Culture	USA	Caged-layer house facilities	None	None
10. Li and Stutzenberger., 2000	2229	<i>Helicobacter pylori</i>	PCR	USA	Dairy Center Poultry Center Swine Center Horse Center Sheep Center	None	None
11. Mian <i>et al.</i> , 2002	2506	<i>Salmonella enteritidis</i> <i>Salmonella</i> spp.	Culture		Food courts Dumping ground Food processing and	None	None

					Poultry farm areas		
12. Alam and Zurek, 2004	3440	<i>Escherichia coli</i> O157:H7	Culture PCR	USA	Beef cattle farm	Ampicillin Apramycin Ceftiofur Chlortetracycline Enrofloxacin Gentamicin Neomycin Oxytetracycline Spectinomycin Trimethoprim-sulfamethoxazole	None
13. Szalanski <i>et al.</i> , 2004	2486	<i>Campylobacter</i> spp. <i>Escherichia coli</i> H7 O157 <i>Escherichia coli</i>	Culture PCR	USA	Turkey facilities.	None	None
14. Rahuma <i>et al.</i> , 2005	150	<i>Escherichia coli</i> <i>Salmonella</i> sp. <i>Shigella sonnei</i> <i>Yersinia enterocolitica</i> <i>Edwardsiella tarda</i> <i>Klebsiella</i> spp. <i>Enterobacter</i> spp. <i>Citrobacter</i> spp. <i>Proteus</i> spp. <i>Providencia</i> spp. <i>Serratia</i> spp. <i>Erwinia</i> spp. <i>Aeromonas</i> spp.	Culture	Libya	Hospital City Streets Abattoir	Amoxicillin Amoxicillin-clavulanic acid Ceftriaxone Cephaloridine Chloramphenicol Amikacin Kanamycin Nalidixic acid Norfloxacin Doxycycline	None

		<i>Flavobacterium</i> spp. <i>Pseudomonas</i> spp. <i>Xanthomonas</i> spp. <i>Weeksella zoohleum</i> <i>Pasteurella</i> spp. <i>Staphylococcus</i> spp. <i>Streptococcus</i> spp.				Trimethoprim– sulphamethoxazole Methicillin Fusidic acid	
15. Nazni <i>et al.</i> , 2005	20	<i>Bacillus</i> spp., <i>Coccobacillus</i> spp. <i>Staphylococcus</i> spp. <i>Micrococcus</i> spp. <i>Streptococcus</i> spp. <i>Acinetobacter</i> spp. <i>Enterobacter</i> spp., <i>Proteus</i> spp. <i>Escherichia</i> spp. <i>Klebsiella</i> spp.	Culture	Malaysia	Food courts Dumping ground Food processing Poultry farm	None	None
16. Macovei and Zurek, 2006	268	<i>Enterococcus gallinarum</i> <i>Enterococcus casseliflavus</i> <i>Enterococcus faecalis</i> <i>Enterococcus faecium</i>	M-PCR	USA	Food restaurant	Tetracycline Chloramphenicol Ciprofloxacin Erythromycin Vancomycin Ampicillin. Streptomycin Kanamycin	<i>tetM</i> <i>tetO</i> , <i>tetW</i> <i>ermB</i>

17. Mawak and Olukose.,2006	200	<i>Escherichia coli</i> <i>Staphylococcus</i> spp. <i>Streptococcus</i> spp. <i>Salmonella</i> spp. <i>Klebsiella</i> spp. <i>Pseudomonas</i> spp. <i>Proteus</i> spp.	Culture	Nigeria	Abattoir Main market Hospital	None	None
18. Nmorsi <i>et al.</i> , 2007	737	<i>Escherichia coli</i> <i>Serratia marcescens</i> <i>Proteus mirabilis</i> <i>Proteus frundi</i> <i>Streptococcus faecalis</i> , <i>Streptococcus pyogenes</i> <i>Klebsiella pneumoniae</i> , <i>Salmonella typhi</i> , <i>Salmonella typhimurium</i> <i>Staphylococcus aureus</i>	Culture	Nigeria	Hospitals, Houses Human habitation Dairy farms Stalls of food Vendors Abattoir Dairy farms	Chloramphenicol, Cloxacillin Tetracycline Streptomycin Oxacillin Clindamycin Ofloxacin	None
19. Forster <i>et al.</i> , 2007	24	<i>Campylobacter jejuni</i> <i>Campylobacter</i> spp. <i>Escherichia coli</i> <i>Enterococci</i> spp. <i>Klebsiella oxytoca</i> <i>Proteus mirabilis</i> <i>Proteus</i> spp. <i>Staphylococcus aureus</i> <i>Staphylococci</i> spp.,	Culture	Germany	Dog pound Poultry house Cattle barn Horse stable Pigpen	None	None

		<i>coagulase negative Streptococci</i> spp.					
20. Barro <i>et al.</i> 2007	1920	<i>Shigella</i> spp. <i>Salmonella</i> spp. <i>Streptococci</i> spp. <i>Staphylococcus aureus</i>	Culture	Burkina Faso	Fruit juice vending site Meat/poultry vending site	None	
21. Macovei <i>et al.</i> , 2008	75	<i>Enterococcus gallinarum</i> <i>Enterococcus casseliflavus</i> <i>Enterococcus faecalis</i> <i>Enterococcus faecium</i>	Culture M-PCR	USA	Cattle feedlot	Tetracycline, Chloramphenicol, Ciprofloxacin, Erythromycin Vancomycin Ampicillin	None
22. Vazirianzadeh <i>et al.</i> , 2008	230	<i>Escherichia coli</i> <i>Staphylococci aureus</i> <i>Pseudomonas</i> spp. <i>Proteus</i> spp.	Culture	Iran	Slaughterhouse Zoo	None	None
23. Vasan <i>et al.</i> , 2008	None	<i>Salmonella typhi</i>	Culture	India	Bus stands Vegetable market Fish market	Ciprofloxacin Chloramphenicol Amikacin Cefpodoxime	
24. Forster <i>et al.</i> , 2009	102	<i>Campylobacter jejuni</i> <i>Campylobacter</i> spp. <i>Escherichia coli</i> <i>Enterococci</i> spp. <i>Klebsiella oxytoca</i> <i>Proteus mirabilis</i> <i>Proteus</i> sp.	Culture	Germany	Cattle barn Dog pound Horse stable Pigpen	None	None

		<i>Staphylococcus aureus</i> <i>Staphylococci</i> spp. CNS					
25. Boumama <i>et al.</i> , 2010	600	<i>Acinetobacter lwoffii</i> <i>Alcaligenes</i> spp. <i>Citrobacter</i> spp. <i>Enterobacter</i> spp. <i>Escherichia coli</i> <i>Klebsiella</i> spp. <i>Leclercia adecarboxylata</i> <i>Moellerella wisconsensis</i> <i>Morganella morganii</i> <i>Pasteurella</i> spp. <i>Proteus</i> spp. <i>Providencia</i> spp. <i>Salmonella</i> spp. <i>Serratia</i> spp. <i>Shigella dysenteriae</i> <i>Yersinia enterocolitica</i> <i>Staphylococcus aureus</i> <i>Staphylococcus coagulase-</i> <i>negative</i> <i>Enterococcus</i> spp.	Culture	Morocco	Residential areas	Ampicillin Cefoxitin Ceftazidime Cefepime Amikacin Amoxicillin- clavulanate Piperacillin- tazobactam Imipenem Ertapenem Meropenem Gentamicin Ciprofloxacin Cotrimoxazole Vancomycin Erythromycin Clindamycin Levofloxacin Oxacillin Penicillin Gentamicin Cotrimoxazole Linezolid Daptomycin	None

26. Davari <i>et al.</i> , 2010	908	<i>Klebsiella pneumoniae</i> <i>Pseudomonas aeruginosa</i> <i>Citrobacter freundii</i> <i>Escherichia coli</i> <i>Bacillus cereus</i> <i>Proteus mirabilis</i>	Culture	Iran	Hospitals Slaughterhouse	Erythromycin Streptomycin Ampicillin Tetracycline Kanamycin Chloramphenicol Co-trimoxazole Gentamicin, Ciprofloxacin Nitrofurantoin Ceftriaxone Cephalexin	None
27. Butler <i>et al.</i> , 2010	None	<i>Acinetobacter baumannii</i> <i>Bacillus cereus</i> <i>Bacillus pumilus</i> <i>Bacillus thuringiensis</i> <i>Cronobacter sakazakii</i> <i>Escherichia coli</i> 0157:H7 <i>Methylobacterium</i> <i>persicinum</i> <i>Shigella dysenteriae</i> <i>Staphylococcus</i> <i>saprophyticus</i> <i>Staphylococcus sciuri</i> <i>Staphylococcus xylosus</i>	Culture PCR	USA	Restaurant	None	None
28. Wang <i>et al.</i> , 2011	220	<i>Salmonella</i> spp.	Culture	Taiwan	Swine Farms	Ampicillin	None

						Amoxicillin– clavulanic acid Chloramphenicol Florfenicol Trimethoprim- sulfamethoxazole Nalidixic acid Ciprofloxacin Streptomycin Gentamicin Ceftriaxone Tetracycline	
29. Choo <i>et al.</i> , 2011	60	<i>Campylobacter. jejuni</i> <i>Campylobacter coli</i> <i>Salmonella hadar</i> <i>Salmonella muenster</i> <i>Salmonella Indiana</i>	Culture	Malaysia	Cafeteria Poultry farm	None	
30. Kassiri <i>et al.</i> , 2012	40	<i>Klebsiella</i> spp. <i>Bacillus</i> spp. <i>Citobacter Diphteroid</i> <i>Staphylococcus</i> spp. <i>Escherichia coli</i> <i>Pseudomonas</i> spp.	Culture	Iran	Hospitals	None	None
31. Ahmed <i>et al.</i> , 2013	40	<i>Escherichia coli</i> <i>Staphylococcus albus</i> <i>Sphylococcus. aureus</i> <i>Klebsiella</i> spp.	Culture	Iraq	Slaughterhouse Garbage dumps Restaurant dumps Hospital dumps	Tetracycline Erythromycin Ciprofloxacin Gentamycin	None

		<i>Salmonella</i> spp. <i>Pseudomonas aeruginosa</i>				Ampicillin Amoxicillin Penicillin Nalidixic acid Ferdantin Amikacin Rifadin	
32. Usui <i>et al.</i> , 2013	91	<i>Escherichia coli</i>	Culture PCR	Japan	Cattle barn	Ampicillin Cefazolin Cefpodoxime Streptomycin Kanamycin Gentamicin Tetracycline Chloramphenicol Nalidixic acid Ciprofloxacin Trimethoprim Fosfomycin Colistin	<i>bla</i> _{TEM} <i>bla</i> _{SHV} <i>bla</i> _{OXA} <i>bla</i> _{CTX-M} <i>bla</i> _{ACC} <i>bla</i> _{FOX} <i>bla</i> _{MOX} <i>bla</i> _{DHA} , <i>bla</i> _{CIT} <i>bla</i> _{EBC} <i>tetA</i> <i>aac(6φ)-Ib-cr</i>
33. Akoh <i>et al.</i> , 2013	150	<i>Enterococci</i> spp.	Culture	Nigeria	Poultry house	Ciprofloxacin Streptomycin Chloramphenicol Ampiclox Rifampicin Norfloxacin Levofloxacin	

34. Doud <i>et al.</i> , 2014	276	<i>Enterococcus faecalis</i> <i>Enterococcus faecium</i>	M-PCR	USA	Wastewater treatment facilities	Doxycycline Gentamicin Erythromycin Ampicillin Ciprofloxacin Vancomycin Nitrofurantoin tigecycline. Streptomycin Tetracycline Linezolid	
35. Al-Yousef., 2014	200	<i>Escherichia coli</i> <i>Helicobacter pylori</i> <i>Staphylococcus aureus</i> <i>Proteus</i> spp. <i>Pseudomonas aeruginosa</i>	Culture	Saudi Arabia	Houses	None	None
36. Blaak <i>et al.</i> , 2014	54	ESBL Producing- <i>Escherichia coli</i>	Culture M-PCR	The Netherlands	Broiler farms	cefotaxime clavulanic acid ceftazidime ceftazidime/clavulanic acid cefoxitin	<i>bla</i> _{OXA} <i>bla</i> _{SHV} <i>bla</i> _{TEM}
37. Davies <i>et al.</i> , 2014	67	<i>Bacillus</i> sp. <i>Clostridia</i> spp. <i>Enterobacteriaceae</i> , <i>Staphylococci</i> spp. <i>Streptococci</i> spp.	Culture	UK	Hospitals	None	None

38. Doud <i>et al.</i> , 2014	None	<i>Enterococcus. faecalis</i>	Culture	USA	Wastewater Treatment Facilities	None	None
39. Cottone <i>et al.</i> , 2014	None	<i>Campylobacter</i> <i>Escherichia coli</i> O157:H7	PCR	USA	Turkey farms	None	None
40. Chaiwong <i>et al.</i> , 2014	1349	<i>Bacillus</i> spp. <i>Citrobacter</i> spp. <i>Coagulase-negative staphylococci</i> <i>Enterobacter</i> spp. <i>Enterococcus</i> spp. <i>Escherichia coli</i> <i>Escherichia coli</i> 0157:H7 <i>Klebsiella</i> spp. <i>Morganella</i> spp. <i>Proteus</i> spp. <i>Providencia</i> spp. <i>Pseudomonas aeruginosa</i> <i>Salmonella</i> spp. <i>Salmonella typhi</i> <i>Shigella</i> spp. <i>Staphylococcus aureus</i> <i>Streptococcus group D non-enterococci</i>	Culture	Thailand	Fresh-food markets Garbage piles Restaurants School cafeterias Paddy fields	None	
41. Hemmatinezhad <i>et al.</i> , 2015	600	<i>Pseudomonas aeruginosa</i>	Culture PCR	Iran	Kitchens Cattle farms Chicken farms Slaughterhouse	Ampicillin Amikacin Carbenicillin Cefalexin	TEM

					Animal hospitals Human hospitals	Ceftazidime, Ceftriaxone Ceftizoxime Cefotaxime Ciprofloxacin, Gentamicin Imipenem/cilastatin Norfloxacin Piperacillin Tobramycin Kanamycin	
42. Solà-Ginés <i>et al.</i> , 2015	615	Extended-Spectrum B-Lactamase-Producing <i>Escherichia coli</i>	Culture	Spain	Broiler farms	Ampicillin, Cefotaxime Ceftazidime Nalidixic acid Ciprofloxacin Gentamicin Streptomycin Kanamycin Chloramphenicol Florfenicol, Trimethoprim, Tetracycline Colistin	<i>bla</i> CTX-M <i>bla</i> SHV <i>bla</i> TEM <i>bla</i> CMY-2 <i>aac(6=)-Ib-cr</i> <i>qnrA</i> <i>qnrB</i> <i>qnrS</i> <i>qepA</i> <i>oqxAB</i>
43. Usui <i>et al.</i> , 2015	74	<i>Escherichia coli</i>	Culture PCR	Japan	Cattle farms	Ampicillin Cefazolin Cefotaxime	<i>tetA</i> <i>tetB</i> <i>tetC</i>

						Kanamycin Gentamicin Dihydrostreptomycin Tetracycline Chloramphenicol Nalidixic acid Enrofloxacin	<i>tetD</i> <i>tetE</i> <i>tetG</i> <i>tetM</i> <i>tetS</i>
44. Ommi <i>et al.</i> , 2015	600	<i>Aeromonas</i> spp.	Culture PCR	Iran	Kitchens Cattle farms Animal hospitals Human hospitals Slaughterhouses	Ampicillin Cefalothin Chloramphenicol Gentamicin Kanamycin Nalidixic acid Tetracycline Trimethoprim- Sulfamethoxazole Amikacin, Carbenicillin Neomycin Ofloxacin Rifampin Tobramycin	None
45. Tsagaan <i>et al.</i> , 2015	267	<i>Escherichia coli</i> <i>Listeria monocytogenes</i> <i>Salmonella</i> spp.	M-PCR	Japan	None	None	None
46. Ghalehnoo, 2015	450	<i>Staphylococcus aureus</i>	Culture	Iran	broiler farm environments.	Ampicillin Ceftriaxone	

						Ciprofloxacin Erythromycin Gentamicin Methicillin Ofloxacin Oxacillin Penicillin Trimethoprim/Sulfa methoxazole Vancomycin	
47. Mohammed <i>et al.</i> , 2016	22	<i>Escherichia coli</i> <i>Enterobacter</i> spp. <i>Klebsiella</i> spp. <i>Staphylococcus aureus</i> <i>Salmonella</i> spp. <i>Shigella</i> spp. <i>Proteus</i> CNS	Culture	Egypt	Livestock farm	Enrofloxacin Cloxacillin Gentamycin Vancomycin Cephalexin, Penicillin G Streptomycin Colistin Clindamycin Tetracycline Cefoxitin Chloramphenicol	None
48. Ranjbar <i>et al.</i> , 2016	600	<i>Klebsiella pneumoniae</i>	Culture PCR	Iran	Kitchens Cattle farms Chicken farms Animal hospitals Huma hospital Slaughterhouses	Ampicillin Amoxicillin Amikacin Cephalexin, Ceftazidime Ceftriaxone Ceftizoxime	None

						Cefotaxime Chloramphenicol Ciprofloxacin Gentamicin Imipenem Nitrofurantoin Piperacillin Tetracycline Kanamycin	
49. Burrus <i>et al.</i> , 2016	35	<i>Escherichia coli</i> O157: H7,	Culture PCR	USA	Dairy farms	None	None
50. Fouda <i>et al.</i> , 2016	None	<i>Aerococcus viridans</i> <i>Pseudomonas fluorescens</i> <i>Kocuria rosea</i> <i>Staphylococcus lentus</i> <i>Micrococcus lentus/lylae</i> <i>Escherichia coli</i> <i>Enterobacter aerogenes</i> <i>Staphylococcus sciuri</i> <i>Staphylococcus lentus</i> <i>Staphylococcus simulans</i> <i>Leuconostoc</i> <i>pseudomesentroides</i> <i>Lactobacillus delbrueckii</i> <i>Pseudomonas veronii</i> <i>Kocuria kristinae</i>	Culture	Egypt	Garbage Decaying organic matters Manure Carrions of different animals	None	None

51. Schaumburg <i>et al.</i> , 2016	895	ESBL-producing <i>Escherichia coli</i> <i>Staphylococci aureus</i>	Culture	Germany	Urban and rural areas	Piperacillin/tazobactam Cefotaxime Ceftazidime Ertapenem Ciprofloxacin Trimethoprim/sulfamethoxazole	None
52. Awache and Forouk.,2016	1059	<i>Escherichia coli</i> <i>Citrobacter freundii</i> , <i>Bacillus megatarium</i> , <i>Bacillus sphaericus</i> <i>Bacillus alvei</i> <i>Moganella morgana</i>	Culture	Nigeria	Cafeteria Food centers	None	None
53. Brits <i>et al.</i> , 2016	10	<i>Bacillus lentus</i> <i>Bacillus pumilus</i> . <i>Bacillus</i> spp. <i>Bacillus subtilis</i> <i>Kurthia gibsonii</i> <i>Kurthia zopfii</i> <i>Myroides profundus</i> <i>Pantoea</i> spp. <i>Proteus mirabilis</i> <i>Pseudomonas</i> spp. <i>Serratia</i> spp. <i>Staphylococcus sciuri</i> <i>Staphylococcus</i> spp.	Culture PCR	South Africa	Municipal refuse	None	

		<i>Williamsia</i> spp.					
54. Songe <i>et al.</i> , 2017	418	Enteropathogenic <i>Escherichia coli</i> <i>Salmonella</i> spp.	Culture PCR	Zambia	Food markets	Ampicillin, Sulfamethoxazole /trimethoprim Streptomycin Ciprofloxacin Tetracycline Gentamicin, Nalidixic acid Chloramphenicol Ceftazidime Norfloxacin Cefotaxime Ceftazidime Cefotaxime	<i>bla</i> _{TEM} <i>bla</i> _{SHV} <i>bla</i> _{CTX-M}
55. Manandhar and Gokhale, 2017	100	<i>Escherichia coli</i> <i>Citrobacter</i> spp. <i>Klebsiella</i> spp. <i>Proteus mirabilis</i> <i>Staphylococcus aureus</i> <i>Enterococcus</i> spp. coagulase <i>Staphylococcus</i> spp.	Culture PCR	Nepal	College canteen ground Kitchen and dining hall	Ampicillin Gentamycin Cefazolin Nitrofurantoin Ciprofloxacin Trimethoprim- Sulphomethoxole Norfloxacin Ofloxacin ceftizoxime Penicillin Amikacin Erythromycin	None

						Cephalexin Ceftriaxone Oxacillin Cefoxitin	
56. Nazari <i>et al.</i> , 2017	300	<i>Enterococci</i> spp. <i>Pseudomonas</i> spp. <i>Proteus</i> spp. <i>Bacillus</i> spp. <i>Staphylococcus aureus</i> <i>Enterobacter</i> spp. <i>Citrobacter</i> spp. <i>Klebsiella</i> spp. <i>Escherichia coli</i>	Culture	Iran	Dumping sites Hospital Fruit and vegetables center Livestock slaughter	Erythromycin Trimethoprim-sulfamethoxazole Gentamycin Ciprofloxacin Nitrofurantoin Cefotaxime Erythromycin Ampicillin Tetracycline Kanamycin Chloramphenicol Cefalotin and cefotaxime	None
57. Omimi <i>et al.</i> , 2017	600	<i>Campylobacter</i> spp. <i>Salmonella</i> spp.	Culture PCR	Iran	Household kitchens Cattle farms Animal hospitals Human hospitals Slaughterhouses Chicken farms	Ampicillin Amoxicillin-clavulanic acid, Aztreonam Ceftazidime Cefalothin, Cefixime Chloramphenicol Ceftriaxone Doxycycline,	None

						Cefotaxime Ciprofloxacin Gentamicin Imipenem/cilastatin Norfloxacin, Kanamycin Nalidixic acid Oxytetracycline Streptomycin Tetracycline Trimethoprim-sulfamethoxazole	
58. Puri-Giri <i>et al.</i> , 2017	463	Non-O157 Shiga Toxin-Producing <i>Escherichia coli</i>	Culture MPCR	USA	Commercial cattle feedlots Dairy farms	None	None
59. Yalli <i>et al.</i> , 2017	150	<i>Escherichia coli</i> <i>Pseudomonas</i> spp <i>Bacillus</i> spp. <i>Enterobacter</i> spp. <i>Staphylococcus</i> spp. <i>Salmonella</i> spp. <i>Proteus</i> spp. <i>Klebsiella</i> spp.	Culture	Nigeria	Kitchens Toilet Rooms	None	None
60. Akter <i>et al.</i> , 2017	15	<i>Escherichia coli</i> <i>Salmonella</i> spp. <i>Shigella</i> spp.	Culture	Bangladesh	Dining room Canteen Hospital	Ampicillin Chloramphenicol Ciprofloxacin	

		<i>Pseudomonas</i> spp. <i>Enterobacter</i> spp. <i>Klebsiella</i> spp.				Gentamycin Imipenem Vancomycin Amoxicillin Penicillin Tetracycline	
61. Yosboonruang <i>et al.</i> , 2018	60	<i>Escherichia coli</i>	Culture PCR	Thailand	Hospital	Ampicillin Amoxicillin/Clavulanic acid Ampicillin/Sulbactam Cephalothin Cefotaxime Meropenem Imipenem) Ciprofloxacin Norfloxacin Amikacin Gentamicin tetracyclines Chloramphenicol Trimethoprim-sulfamethoxazole	<i>intl</i>
62. Zhang <i>et al.</i> , 2018	252	<i>Escherichia coli</i> <i>Providencia stuartii</i> <i>Enterococcus faecium</i> . <i>Staphylococcus aureus</i>	Culture qPCR	China	University campus	Colistin	<i>mcr-1</i> <i>mcr-2</i> <i>mcr-3</i>

		<i>Klebsiella pneumoniae</i> <i>Citrobacter freundii</i> <i>Acinetobacter baumannii</i> <i>Pseudomonas aeruginosa</i> : <i>Enterobacter species</i> <i>Enterobacter ludwigii</i>					
63. Alves <i>et al.</i> , 2018	20	<i>Escherichia coli</i>	Culture PCR	Brazil	Dairy farm environment	Ampicillin amoxicillin/clavula nic acid cephalexin cefoperazone ceftiofur ceftriaxone chloramphenicol enrofloxacin ciprofloxacin gentamicin sulfamethoxazole /trimethoprim tetracycline	<i>ampC</i> <i>bla</i> _{TEM} <i>bla</i> _{SHV} <i>bla</i> _{CRX-M} <i>tetA</i> <i>int1</i>
64. Baker <i>et al.</i> , 2018	150	<i>Escherichia coli</i> <i>Salmonella typhi</i> <i>Proteus mirabilis</i> <i>Staphylococcus aureus</i> <i>Klebsiella pneumoniae</i> <i>Shigella</i> spp. <i>Citrobacter freundii</i> <i>Hafina</i> spp.	Culture	Iraq	Vegetable market Butchers' market Sheep sheds.	Amikacin Chloramphenicol Erythromycin Trimethoprim Sulfamethoxazol Amoxycillin Tetracyclin Ciprofloxacin	None

		<i>Serratia marcescens</i>				Ciprofloxacin Vancomycin Gentamycin Oxacillin	
65. Cervelina <i>et al.</i> , 2018	200	<i>Escherichia coli</i> <i>Salmonella</i> spp.	Culture	Brazil	Swine production farms	Ampicillin Cefalotin Ciprofloxacin Norfloxacin	None
66. Paulson, 2018	100	<i>Pseudomonas</i> spp. <i>Klebsiella</i> spp. <i>Staphylococcus</i> spp. <i>Escherichia coli</i> <i>Streptococcus</i> spp. <i>Enterobacter</i> spp. <i>Salmonella</i> spp. <i>Bacillus</i> spp.	Culture	Gujarat	Fish market Butcherries Vegetable market Residential area	Tricarillin Meropenem Levofloxacin Moxifloxacin Cefprozil Cefpirome Ceftizoxime Cefpodoxime Cefoparazone Piperacillin Tobramycin Sparfloxacin Gatifloxacin Imipenem	None
67. Pohlenz <i>et al.</i> , 2018	224	Shiga-toxigenic <i>Escherichia coli</i>	Culture	USA	Restaurant area	None	None
68. Ibrahim <i>et al.</i> , 2018	27	<i>Escherichia coli</i> <i>Salmonella</i> spp.	Culture	Nigeria	Dumpsite Canteen	None	None

		<i>Pseudomonas</i> spp. <i>Shigella</i> spp. <i>Klebsiella</i> spp. <i>Staphylococcus</i> spp. <i>Streptococcus</i> spp. <i>Bacillus</i> spp. <i>Proteus</i> spp.			Indoor premise		
69. Deakpe <i>et al.</i> , 2018	210	<i>Staphylococcus</i> spp. <i>Bacillus</i> spp. <i>Klebsiella</i> spp. <i>Escherichia</i> spp. <i>Proteus</i> spp. <i>Salmonella</i> spp. <i>Moraxella</i> spp.	Culture	Nigeria	Wurukum Market Modern Market Railway Market Kwararafa Quarters High Level Market North Bank Market Wadata Market	None	
70. Poude <i>et al.</i> , 2019	320	<i>Escherichia coli</i> <i>Klebsiella pneumoniae</i> <i>Staphylococcus</i> spp.	Culture PCR	USA	Dairy unit Dog kennel Poultry farm Beef cattle unit Urban trash facility Urban downtown area	Ampicillin Amoxicillin- clavulanic acid Ceftazidime Amikacin Gentamicin Streptomycin, Tetracycline Doxycycline Chloramphenicol Ciprofloxacin	bla _{AMPH} bla _{AMPC1} bla _{AMPC2} FloR bla _{MRDA} , StrA StrB SuII bla _{TEM-1D} tetA Aac3-IIa bla _{AMPH}

						Trimethoprim-sulfamethoxazole Cefoxitin and oxacillin	<i>bla</i> _{CTXM-1}
71. Sobur <i>et al.</i> , 2019b	60	colistin-resistant <i>Escherichia coli</i>	Culture PCR	Bangladesh	Poultry farm	Colistin Imipenem Meropenem Ertapenem	<i>mcr-3</i>
72. Sobur <i>et al.</i> , 2019a	60	<i>Salmonella</i> spp.	Culture PCR	Bangladesh	Fish market	Ampicillin, Azithromycin Chloramphenicol, Ciprofloxacin, Gentamicin, Oxytetracycline, Tetracycline, Streptomycin, Imipenem, Meropenem	<i>tetA</i> , <i>tetB</i> <i>qnrA</i>
73. Neupane <i>et al.</i> , 2019	60	<i>Mannheimia haemolytica</i> <i>Pasteurella multocida</i> <i>Histophilus somni</i>	Culture PCR	USA	Commercial cattle operation	None	None
74. Galal <i>et al.</i> , 2019	None	<i>Escherichia coli</i> <i>Bacillus subtilis</i> <i>Pseudomonas aeruginosa</i>	None	Saudi Arabia	Sakaka city	None	

75. Nwankwo <i>et al.</i> , 2020	150	<i>Escherichia coli</i> <i>Klebsiella</i> spp. <i>Staphylococcus aureus</i> <i>Proteus vulgaris</i> <i>Proteus mirabilis</i> <i>Pseudomonas aeruginosa</i> <i>Salmonella</i> spp. <i>Enterococcus faecalis</i> <i>Staphylococcus aureus</i> <i>Staphylococcus epidermidis</i> <i>Streptococcus</i> spp.	Culture	Nigeria	Market Refuse Broken sewage Restaurant Clinic	Ofloxacin Pefloxacin Ceftriaxone Amoxicillin Clavulanate Gentamicin Streptomycin Cephalexin, Cotrimoxazole Ampicillin	None
76. Akter <i>et al.</i> , 2020	140	<i>Staphylococcus aureus</i> <i>Salmonella</i> spp. <i>Escherichia coli</i>	Culture PCR	Bangladesh	Veterinary Hospital Poultry farms University Canteens Restaurants Households Dairy farms College hospital	Ciprofloxacin Ceftriaxone Chloramphenicol Gentamycin Azithromycin Nalidixic acid Streptomycin Erythromycin Tetracycline Penicillin	<i>tetA</i> , <i>tetB</i> , <i>mcr-3</i> , <i>mecA</i> , <i>mecC</i>
77. Kababian <i>et al.</i> , 2020	160	<i>Staphylococcus epidermidis</i> <i>Streptococcus pneumoniae</i> <i>Staphylococcus aureus</i> <i>Enterococcus</i> spp. <i>Klebsiella oxytoca</i>	Culture	Iran	Animal husbandry	None	

		<i>Proteus mirabilis</i> <i>Proteus vulgaris</i> <i>Serratia marcescens</i> <i>Escherichia coli</i> <i>Klebsiella pneumoniae</i> <i>Pseudomonas aeruginosa</i>					
78. Odetoyin <i>et al.</i> , 2020	25	<i>Klebsiella pneumoniae</i> <i>Escherichia coli</i> <i>Staphylococcus aureus</i> <i>Pseudomonas aeruginosa</i> <i>Proteus spp</i> <i>Salmonella spp</i>	Culture	Nigeria	Slaughterhouse Garbage Dump Eatery Hospital Human House	Pefloxacin Gentamycin Amoxicillin Ciprofloxacin Streptomycin Cotrimoxazole Chloramphenicol Sparfloxacin Augmentin Ofloxacin	

CNS: Coagulase Negative Streptococci M-PCR: Multiplex-Polymerase chain reaction PCR: Polymerase chain reaction USA: United states of America, UK: United Kingdom

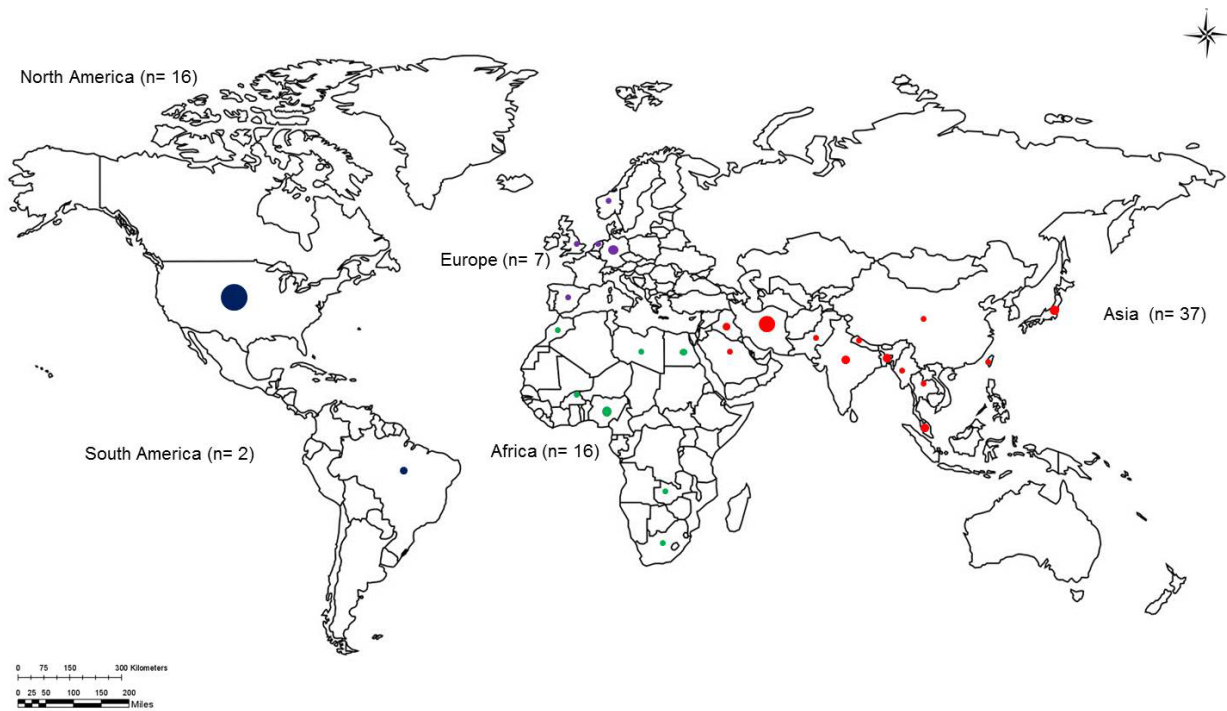


Figure 3.2 The number of countries, number of studies and sample size per continent. The size of the circle indicates the number of studies conducted in that country with different colors for different continents whereby green is for Africa, red for Asia, blue for North America and black is for South America.

3.3.3 META-ANALYSIS

A total of 44 eligible studies were included in the quantitative synthesis (Table 3.2), this was owing to the study being definite with bacterial species. Substantial heterogeneity was observed between the eligible studies. The pooled estimates and heterogeneities of the eligible studies are presented in Tables 3.3 to 3.7 for the five selected pathogenic bacteria including *Escherichia coli*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In all meta-analyses conducted, the overall effect estimates and their corresponding 95% confidence intervals (CI) did not overlap the point estimate (Figure 3.3 to 3.7). Similarly, the latter was observed in all the subgroups (Figure 3.8-3.11).

Table 3.2 A meta-analysis of eligible studies reporting on selected pathogenic bacteria isolated from houseflies in different regions

Study reference	Region	Method of diagnosis utilized	Sample size	<i>E. coli</i> No. of positives: (Prevalence)	<i>E. faecium</i> No. of positives: (Prevalence)	<i>S. aureus</i> No. of positives: (Prevalence)	<i>K. pneumoniae</i> No. of positives: (Prevalence)	<i>P. aeruginosa</i> No. of positives: Prevalence
1. Rosef and Kapperud, 1983	Norway	Culture	608	-	-	-	-	-
2. Fotedar <i>et al.</i> , 1992a	India	Culture	113	22:(19.5%)	-	24:(21.2%)	-	5:(4.4%)
3. Fotedar <i>et al.</i> , 1992b	India	Culture	240	-	-	-	45:(18.8%)	-
4. Moriya <i>et al.</i> , 1999	Japan	Culture PCR	89	10:(11.2%)	-	-	-	-
5. Szalanski <i>et al.</i> , 2004	USA	Culture PCR	2486	160:(6.4%)	-	-	-	-
6. Alam and Zurek, 2004	USA	Culture PCR	3440	75:(2.2%)	-	-	--	-
7. Rahuma <i>et al.</i> , 2005	Libya	Culture	150	58:(38.7%)	-	-	-	-
8. Macovei and Zurek, 2006	USA	PCR	268	-	14:(5.2%)	-	-	-
9. Mawak and Olukose., 2006	Nigeria	Culture	200	30:(15%)	-	-	-	-
10. Nmorsi <i>et al.</i> , 2007	Nigeria	Culture	737	194:(26.3%)	-	-	-	-
11. Macovei <i>et al.</i> , 2008	USA	Culture PCR	75	-	3:(4%)	-	-	-
12. Vazirianzadeh <i>et al.</i> , 2008	Iran	Culture	230	230:(100%)	-	169:(73.5%)	-	-
13. Bouamama <i>et al.</i> , 2010	Morocco	Culture	600	26:(4.3%)	-	8:(1.3%)	-	-
14. Davari <i>et al.</i> , 2010	Iran	Culture	908	45:(4.95%)	-	-	-	77:(8.5%)
15. Choo <i>et al.</i> , 2011	Malaysia	Culture	60	-	-	-	-	-

16. Usui <i>et al.</i> , 2013	Japan	Culture PCR	91	41:(45.1%)	-	-	-	-
17. Al-Yousef., 2014	Saudi Arabia	Culture	200	85:(42,5%)	-	21:(10.5%)	-	22:(11%)
18. Doud <i>et al.</i> , 2014	USA	PCR	276	-	60:(21.7%)	-	-	-
19. Chaiwong <i>et al.</i> , 2014	Thailand	culture	1349	94:(6.96%)	-	13:(0.96%)	-	97:(7.2%)
20. Hemmatinezhad <i>et al.</i> , 2015	Iran	Culture PCR	600	-	-	-	-	53:(8.8%)
21. Solà-Ginés <i>et al.</i> , 2015	Spain	Culture	615	41:(6.7%)	-	-	-	-
22. Usui <i>et al.</i> , 2015	Japan	Culture PCR	74	13:(17.6%)	-	-	-	-
23. Tsagaan <i>et al.</i> , 2015	Japan	PCR	267	114:(42.7%)	-	-	-	-
24. Ghalehnoo,2015	Iran	Culture	450	-	-	87:(19.3%)	-	-
25. Mohammed <i>et al.</i> , 2016	Egypt	Culture	22	6:(27.3%)	-	2:(9.1%)	-	-
26. Ranjbar <i>et al.</i> , 2016	Iran	Culture PCR	600	-	-	-	68:(11.3%)	-
27. Burrus <i>et al.</i> , 2016	USA	Culture PCR	35	27:(77.1%)	-	-	-	-
28. Awache and Forouk.,2016	Nigeria	Culture	1059	177: (16.7%)	-	-	113:(10.7%)	-
29. Brits <i>et al.</i> , 2016	South Africa	Culture PCR	10	-	-	-	-	-
30. Songe <i>et al.</i> , 2017	Zambia	Culture PCR	418	307:(73.4%)	-	-	-	-
31. Manandhar and Gokhale, 2017	Nepal	Culture PCR	100	8:(8%)	-	4:(4%)	-	-

32. Nazari <i>et al.</i> , 2017	Iran	Culture	300	46:(15.3%)	-	25; (8.3%)	-	-
33. Puri-Giri <i>et al.</i> , 2017	USA	Culture PCR	463	159:(34.3%)	-	-	-	-
34. Yalli <i>et al.</i> , 2017	Nigeria	Culture	150	11: (7.3%)	-	12:(8%)	-	-
35. Akter <i>et al.</i> , 2017	Banglade sh	Culture	15	15:(100%)	-	-	-	-
36. Yosboonruang <i>et al.</i> , 2018	Thailand	Culture PCR	60	60:(100%)	-	-	-	-
37. Baker <i>et al.</i> , 2018	Iraq	Culture	150	36:(24%)	-	25:(1.3%)	11:(7.3%)	-
38. Pohlenz <i>et al.</i> , 2018	USA	Culture	224	205:(91.1%)	-	-	-	-
39. Sobur <i>et al.</i> , 2019a	Banglade sh	Culture PCR	300	40:(13.3%)	-	-	-	-
40. Neupane <i>et al.</i> , 2019	USA	Culture PCR	60	-	-	-	-	-
41. Nwankwo <i>et al.</i> , 2020	Nigeria	Culture	150	35: (23.3%)	-	23:(15.3%)	-	9:(6%)
42. Akter <i>et al.</i> , 2020	Banglade sh	Culture PCR	140	72:(51.4%)	-	110:(78.6%)	-	-
43. Kababian <i>et al.</i> , 2020	Iran	Culture	160	59:(36.9%)	-	27:(16.9%)	-	33:(20.6%)
44. Odetoyin <i>et al.</i> , 2020	Nigeria	Culture	25	25:(100%)	-	11:(44%)	17:(68%)	3:(12%)

USA: United State of America

3.3.3.1 Pooling and heterogeneity estimates

Pooled proportions and their corresponding heterogeneities are presented in Table 3.3 – 3.7 for *Escherichia coli*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus* respectively. Substantial heterogeneity was observed for the selected pathogens as well as in the subgroup analysis. For *E. coli*, high heterogeneity was observed ($Q = 10739.5523$; $I^2 = 99.60$; 95%CI: 99.57 - 99.63; $Q-p < 0.0001$), followed by *S. aureus* ($Q = 2439.1220$; $I^2 = 98.24$; 95%CI: 97.99 - 98.45; $Q-p < 0.0001$), *P. aeruginosa* ($Q = 1283.2227$; $I^2 = 96.65$; 95%CI: 96.07 - 97.15; $Q-p < 0.0001$), *K. pneumoniae* ($Q = 1576.6073$; $I^2 = 97.27$; 95%CI: 96.83-97.65; $Q-p < 0.0001$) and *E. faecium* ($Q = 317.6145$; $I^2 = 86.46$; 95%CI: 82.70 - 89.41; $Q-p < 0.0001$) being the least. The forest plot depicting the point estimate for individual studies reporting the occurrence of *E. coli*, *E. faecium*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus* are graphically presented (Figure 3.3-3.7).

Table 3.3 The proportion of *Escherichia coli* isolated from houseflies sampled from regions, screening methods and sampling locations

	Number of studies	Pooled estimate		Measure of heterogeneity		
		Sample Size	Positive	Q	I ² (95%CI)	Significance level Q-p
Bacterial species						
<i>Escherichia coli</i>	44	20455	2711	10739.5523	99.60 (99.57-99.63)	< 0.0001
Region						
Africa	11	13581	875	3569.6776	99.72 (99.68-99.76)	< 0.0001
America	9	5097	41	2599.8385	99.69 (99.64-99.74)	< 0.0001
Asia	22	196	1003	1487.0151	98.59 (98.32-98.81)	< 0.0001
Europe	2	2	717	279.5474	99.64 (99.42-99.78)	< 0.0001
Sampling Locations						
Dumping Sites	3	9906	17	86.2819	97.68 (95.57-98.79)	< 0.0001
Farms	18	1705	797	2075.0757	99.18 (99.04-99.30)	< 0.0001
Hospitals	13	634	213	738.0189	98.37 (97.93-98.72)	< 0.0001
Market	4	393	351	40.9600	92.68 (84.47-96.55)	< 0.0001
No specific location	3	509	155	13.6833	85.38 (57.08-95.02)	= 0.0011
Restaurant	6	493	245	6303.3062	99.75 (99.72-99.77)	< 0.0001
Slaughterhouse (Abattoir)	7	253	94	206.0165	97.09 (95.62-98.06)	< 0.0001
University Campus/Residential houses	7	426	186	134.0283	95.52 (92.86-97.19)	< 0.0001
Wastewater/Broken- Sewage	2	388	9	35.1989	97.16 (92.69 - 98.90)	< 0.0001
Diagnostic technique						

Culture	25	319	1537	2698.8736	99.11 (98.98-99.23)	< 0.0001
PCR	2	549	114	173.0078	99.42 (98.97-99.67)	< 0.0001
Culture and PCR	17	11587	985	6303.3062	99.75 (99.72-99.77)	< 0.0001

Table 3.4 The proportion of *Enterococcus faecium* isolated from houseflies sampled from regions, screening methods and sampling locations

	Number of studies	Pooled estimate		Measure of heterogeneity		
		Sample Size	Positive	Q	I ² (95%CI)	Significance level Q-p
Bacterial species						
<i>Enterococcus faecium</i>	44	20455	77	317.6145	86.46 (82.70-89.41)	< 0.0001
Region						
America	9	1581	77	178.3214	95.51 (93.27-97.01)	< 0.0001
Sampling Locations						
Dumping Sites	3	9906	4	20.4559	90.22 (74.03-96.32)	< 0.0001
Farms	18	1705	3	15.6957	00.00 (00.00-45.86)	= 0.5455
Restaurant	17	11587	14	25.1695	36.43 (00.00-64.46)	= 0.0669
Wastewater/Broken- Sewage	2	388	60	13.7057	92.70 (75.50-97.83)	= 0.0002
Diagnostic technique						
Culture	25	7970	60	228.5404	89.85 (86.19-92.54)	< 0.0001
PCR	2	549	14	30.1038	96.68 (91.08-98.76)	< 0.0001
Culture and PCR	17	11587	3	25.1695	36.43 (00,00-64.46)	= 0.0669

Table 3.5 Proportion of *Klebsiella pneumoniae* isolated from houseflies sampled from regions, screening methods and sampling locations

	Number of studies	Pooled estimate		Measure of heterogeneity		
		Sample Size	Positives	Q	I ² (95%CI)	Significance level Q-p
Bacterial species						
<i>Klebsiella pneumoniae</i>	44	20455	279	1576.6073	97.27 (96.83-97.65)	< 0.0001
Region						
Africa	11	13581	17	79.1374	87.36 (79.31-92.28)	< 0.0001
Asia	22	196	262	1314.0651	98.40 (98.09-98.67)	< 0.0001
Sampling Locations						
Farms	18	1705	51	262.1432	93.51 (91.14-95.26)	< 0.0001
Hospitals	13	634	164	487.8350	97.54 (96.76-98.13)	<0.0001
Restaurant	6	493	2	628.8196	97.46 (96.76-98.00)	< 0.0001
Slaughterhouse (Abattoir)	7	253	37	91.3336	93.43 (88.91-96.11)	< 0.0001
University Campus/Residential houses	7	426	4	17.1543	65.02 (21.35-84.45)	= 0.0087
Diagnostic technique						
Culture	25	319	211	889.4596	97.30 (97.70-97.79)	< 0.0001
Culture and PCR	17	11587	68	628.8196	97.46 (96.76-98.00)	< 0.0001

Table 3.6 The proportion of *Pseudomonas aeruginosa* isolated from houseflies sampled from regions, screening methods and sampling locations

	Number of studies	Pooled estimate		Measure of heterogeneity		
		Sample Size	Positives	Q	I ² (95%CI)	Significance level Q-p
Bacterial species						
<i>Pseudomonas aeruginosa</i>	44	20455	299	1283.2227	96.65 (96.07-97.15)	< 0.0001
Region						
Africa	11	13581	12	52.8707	81.09 (67.19-89.10)	< 0.0001
Asia	22	196	287	772.7609	97.28 (96.63-97.81)	< 0.0001
Sampling Locations						
Dumping Sites	3	9906	5	23.9932	91.66 (78.72-96.74)	< 0.0001
Farms	18	1705	53	235.3866	92.78 (90.03-94.77)	< 0.0001
Hospitals	13	634	107	282.6736	95.75 (94.12-96.94)	< 0.0001
Slaughterhouse (Abattoir)	7	253	7	51.2794	88.30 (78.30-93.69)	< 0.0001
University Campus/Residential houses	7	426	22	31.5330	80.97 (61.56-90.58)	< 0.0001
Wastewater/Broken- Sewage	2	388	4	14.9064	93.29 (78.01-97.95)	= 0.0001
Diagnostic technique						
Culture	25	319	246	595.1690	95.97 (94.94-96.79)	< 0.0001
Culture and PCR	17	11587	53	490.0658	96.74 (95.76-97.48)	< 0.0001

Table 3.7 The proportion of *Staphylococcus aureus* isolated from houseflies sampled from regions, screening methods and sampling locations.

	Number of studies	Pooled estimate		Measure of heterogeneity		
		Sample Size	Positives	Q	I ² (95%CI)	Significance level Q-p
Bacterial species						
<i>Staphylococcus aureus</i>	44	20455	561	2439.1220	98.24 (97.99-98.45)	< 0.0001
Region						
Africa	11	13581	56	230.0741	95.65 (93.77-96.97)	< 0.0001
Asia	22	196	505	1414.2219	98.52 (98.23-98.76)	< 0.0001
Sampling Locations						
Dumping Sites	3	9906	7	33.2093	93.98 (85.80-97.47)	< 0.0001
Farms	18	1705	132	777.4544	97.81 (97.27-98.25)	< 0.0001
Hospitals	13	634	31	79.6692	84.94 (75.80-90.62)	< 0.0001
Market	4	393	4	17.0149	82.53 (54.61-93.15)	= 0.0007
No specific location	3	509	12	38.1214	94.75 (88.02-97.70)	< 0.0001
Restaurant	6	493	37	518.4722	96.91 (96.01-97.61)	< 0.0001
Slaughterhouse (Abattoir)	7	253	3	13.5396	55.69 (00.00-80.97)	= 0.0352
University Campus/Residential houses	7	426	44	42.3304	85.83 (72.83-92.61)	< 0.0001
Wastewater/Broken- Sewage	2	388	6	22.6646	95.59 (87.18-98.48)	< 0.0001
Diagnostic technique						
Culture	25	319	447	1603.7790	98.50 (98.23-98.73)	< 0.0001
Culture and PCR	17	11587	114	518.4722	96.91 (96.01-97.61)	< 0.0001

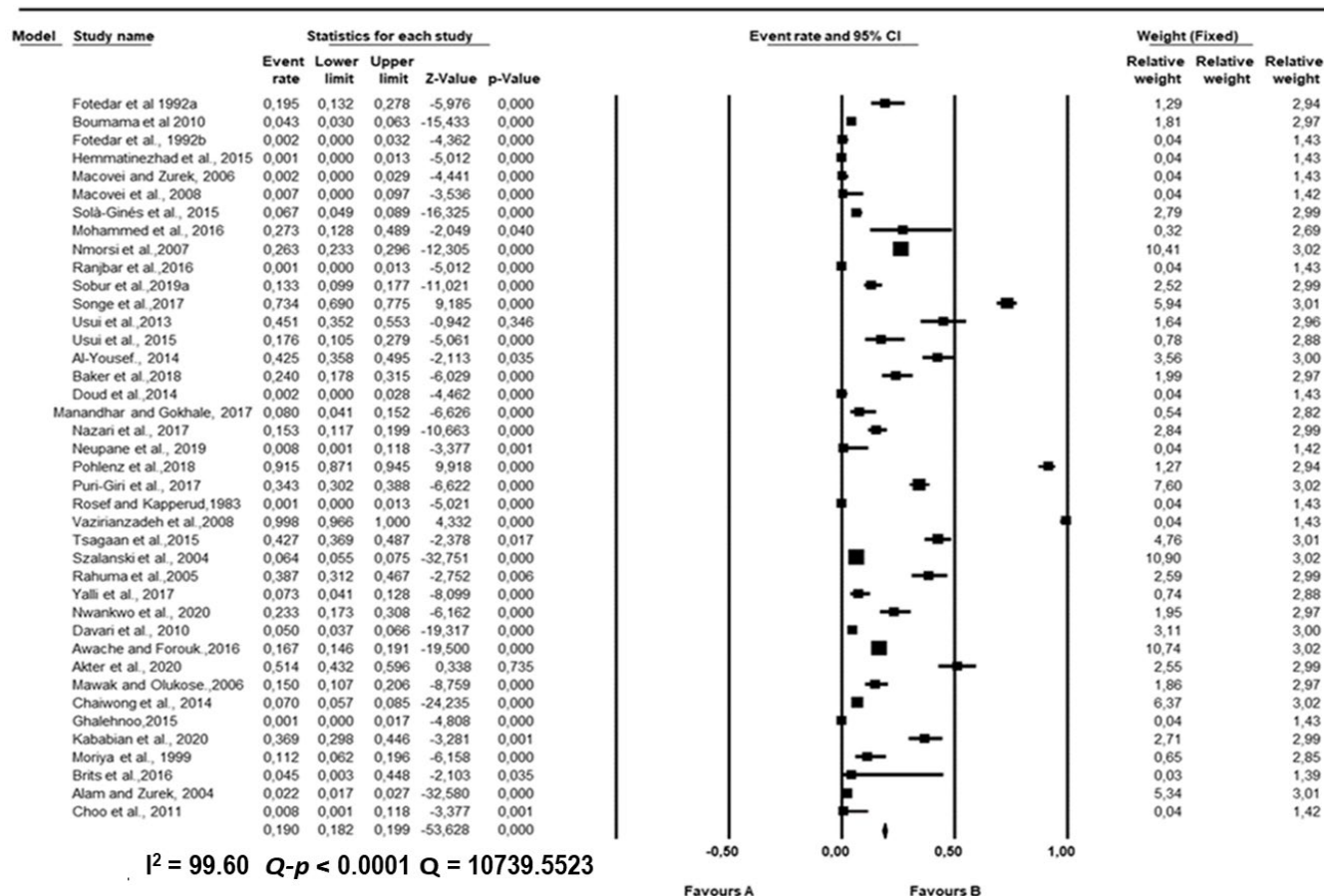


Figure 3.3 Forest plot showing the pooled estimates of *Escherichia coli* proportion isolated from houseflies. The squares demonstrate the individual point estimate. The diamond at the base indicates the pooled estimates from the overall studies.

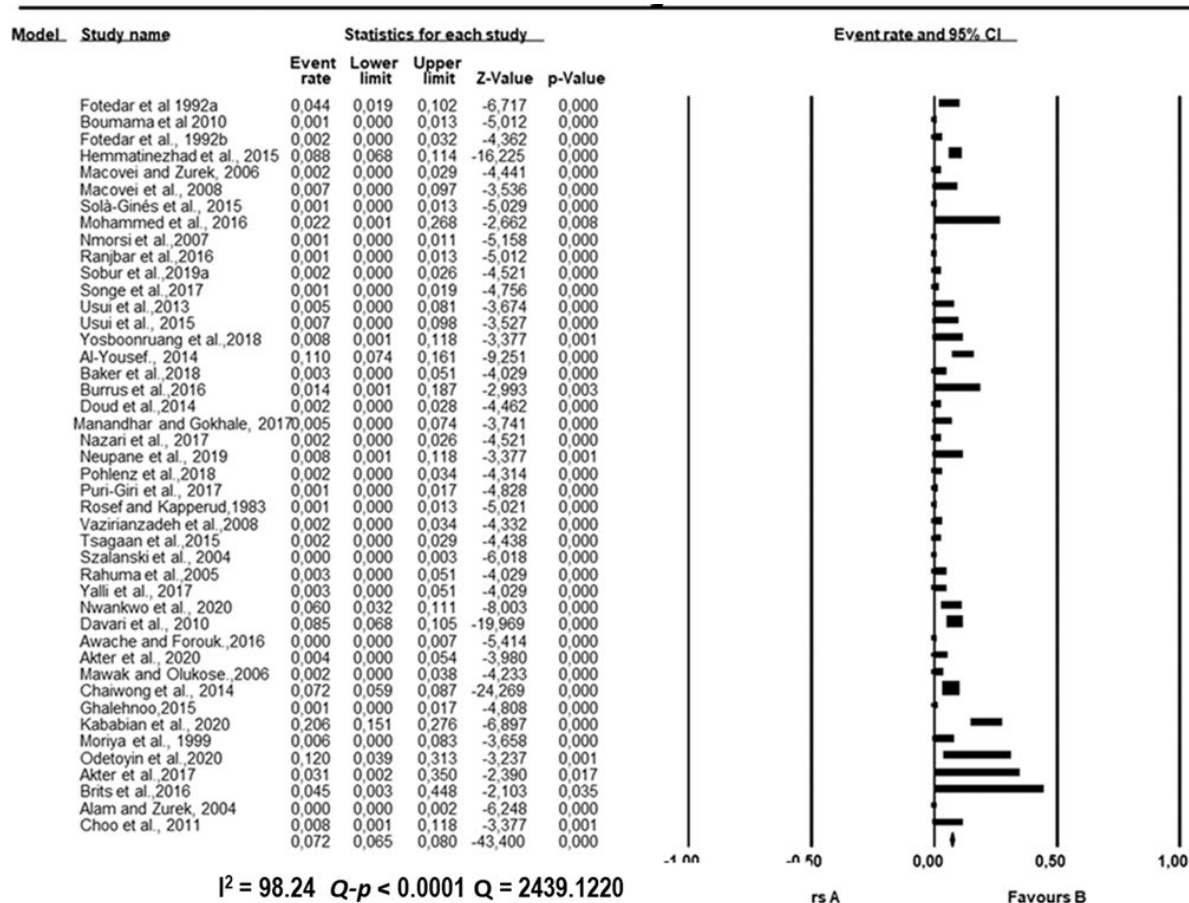


Figure 3.4 Forest plot showing the pooled estimates of *Staphylococcus aureus* proportion isolated from houseflies. The squares demonstrate the individual point estimate. The diamond at the base indicates the pooled estimates from the overall studies.

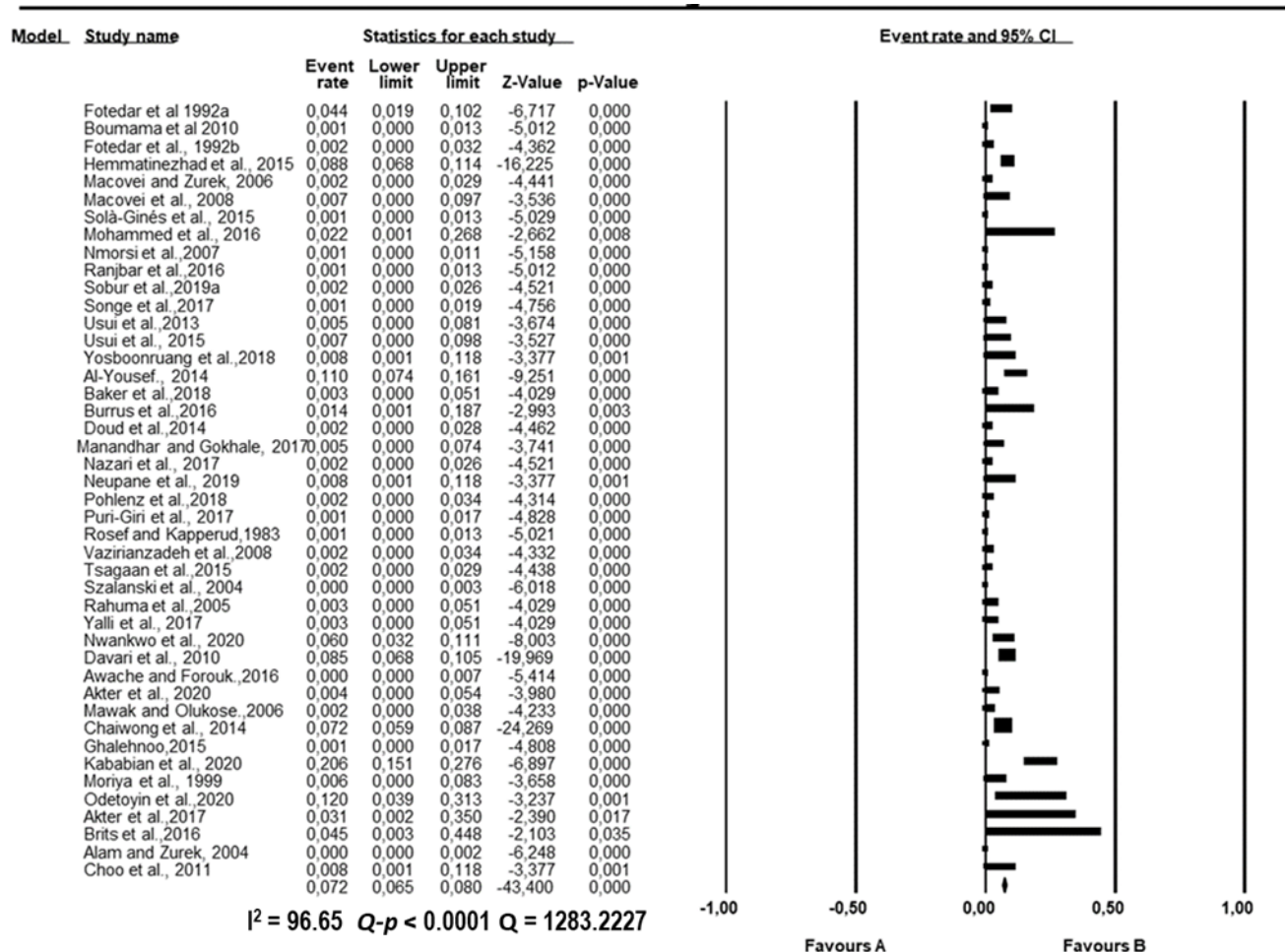


Figure 3.5 Forest plot showing the pooled estimates of *Pseudomonas aeruginosa* proportion isolated from houseflies. The squares demonstrate the individual point estimate. The diamond at the base indicates the pooled estimates from the overall studies.

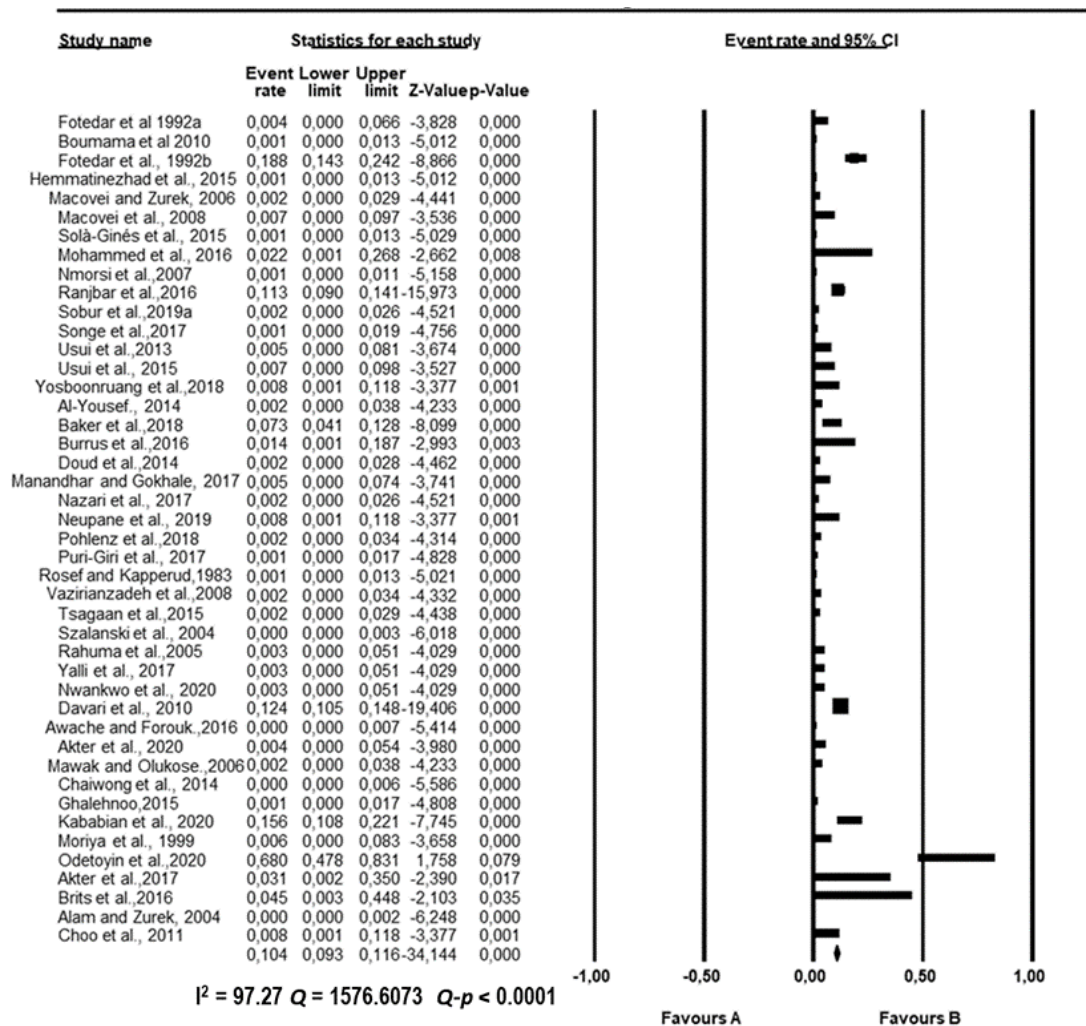


Figure 3.6 Forest plot showing the pooled estimates of *Klebsiella pneumoniae* proportion isolated from houseflies. The squares demonstrate the individual point estimate. The diamond at the base indicates the pooled estimates from the overall studies

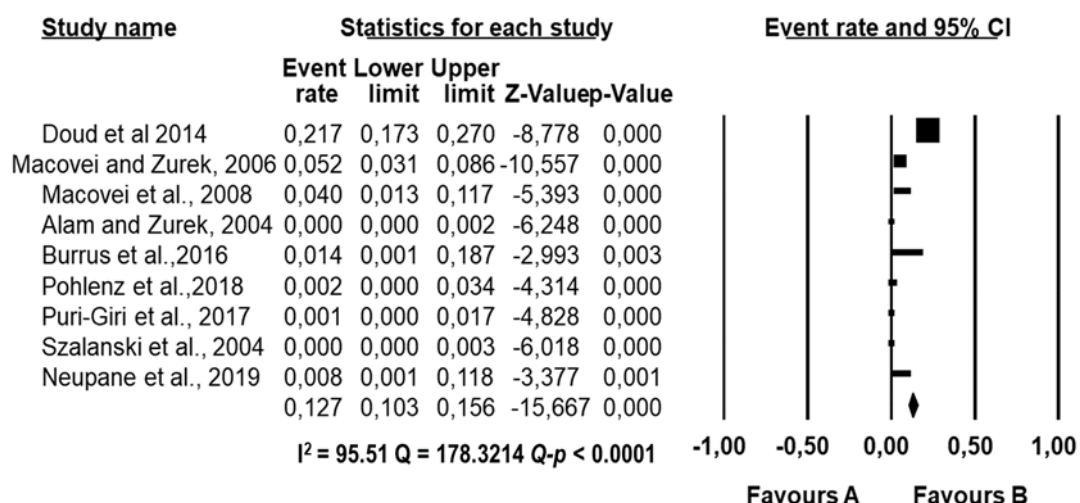


Figure 3.7 Forest plot showing the pooled estimates of *Enterococcus faecium* proportion isolated from houseflies. The squares demonstrate the individual point estimate. The diamond at the base indicates the pooled estimates from the overall studies.

3.3.3.2 Subgroup analysis

Subgroup analyses were done for study locations (Africa, America, Asia and Europe), study sites (Dumping sites, farms, hospitals, market, no specific location, restaurant, slaughterhouse (Abattoir), university campus/residential houses and wastewater/broken sewage), and diagnosis methods used (culture and molecular techniques [PCR]). Different subgroup analysis was undertaken for the five selected bacteria. The forest plot showing the point estimates results of the subgroup analysis are depicted in Figure 3.8- 3.11.

3.3.3.3 Prevalence based on study region

High degree of heterogeneity with respect to *E. coli* was observed in four continents whereas other pathogens including *P. aeruginosa* and *S. aureus* were observed in both Africa and Asia whilst *E. faecium* was observed only in America (Table 3.3 – 3.7). With regards to *E. coli* identified in Africa, a high degree of heterogeneity was observed ($Q = 3569.6776$; $I^2 = 99.72$; 95%CI: 99.68 - 99.76; $Q-p < 0.0001$) followed by America with ($Q = 2599.8385$; $I^2 = 99.69$; 95%CI: 99.64 - 99.74; $Q-p < 0.0001$), while the least observed was in Asia ($Q = 1487.0151$; $I^2 = 98.59$; 95% CI: 98.32 - 98.81; $Q-p < 0.0001$) (Table 2). The results were at variance for *S. aureus* and *P. aeruginosa* with high heterogeneity ($Q = 1414.2219$; $I^2 = 98.52$; 95%CI: 98.23 - 98.76; $Q-p < 0.0001$) and ($Q = 772.7609$; $I^2 = 97.28$; 95%CI: 96.63 - 97.81; $Q-p < 0.0001$) in Asia.

3.3.3.4 Prevalence based on sampling locations

Heterogeneity was found among studies investigating different pathogens in different locations (Table 3.2-3.6).

Farms: High heterogeneity was found among studies investigating *E. coli*, *S. aureus* and *P. aeruginosa* ($Q = 2075.0757$; $I^2 = 99.18$; 95%CI: 99.04-99.30; $P < 0.0001$), ($Q = 777.4544$; $I^2 = 97.81$; 95%CI: 97.27 - 98.25; $Q-p < 0.0001$) and ($Q = 235.3866$; $I^2 = 92.78$; 95%CI: 90.03 - 94.77; $Q-p < 0.0001$) respectively. However, *K. pneumoniae* ($Q = 262.1432$; $I^2 = 93.51$; 95%CI: 91.14-95.26; $P < 0.0001$) was the least as compared with other pathogens.

Hospitals: Four pathogens namely *E. coli*, *P. aeruginosa*, *K. pneumoniae* ($Q = 738.0189$; $I^2 = 98.37$; 95%CI: 97.93-98.72; $Q-p < 0.0001$), ($Q = 282.6736$; $I^2 = 95.75$; 95%CI: 94.12 - 96.94; $Q-p < 0.0001$) and ($Q = 487.8350$; $I^2 = 97.54$; 95%CI: 96.76-98.00; $Q-p < 0.0001$) were found to have high heterogeneity respectively, with *S. aureus* being the least.

Dumping site: High heterogeneity was observed from the studies investigating four pathogens including *E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecium*, ($Q = 86.2819$; $I^2 = 97.68$; 95%CI: 95.57-98.79; $Q-p < 0.0001$), ($Q = 33.2093$; $I^2 = 93.98$; 95%CI: 85.50 - 97.47; $Q-p < 0.0001$), ($Q = 23.9932$; $I^2 = 91.66$; 95%CI: 78.72 - 96.74; $Q-p < 0.0001$) and ($Q = 20.4559$; $I^2 = 90.22$; 95%CI: 74.03-96.32; $Q-p < 0.0001$) respectively.

Market: only two pathogens namely, *E. coli* and *S. aureus* were identified from the houseflies sampled from the marketplace with high heterogeneity ($Q = 40.9600$; $I^2 = 92.68$; 95%CI: 84.47 - 96.55; $Q-p < 0.0001$) and ($Q = 17.0149$; $I^2 = 82.53$; 95%CI: 54.61 - 93.15; $Q-p < 0.0007$) respectively.

Restaurant: High heterogeneity was observed among studies investigating *E. coli* and *K. pneumoniae* ($Q = 518.4722$; $I^2 = 92.91$; 95%CI: 96.01 - 97.61; $Q-p < 0.0001$) and ($Q = 628.8196$; $I^2 = 97.46$; 95%CI: 96.76 - 98.00; $Q-p < 0.0001$).

Slaughterhouse/abattoir: High heterogeneity was observed among studies investigating *E. coli*, *K. pneumoniae* and *P. aeruginosa* ($Q = 206.0165$; $I^2 = 97.09$; 95%CI: 95.62-98.06; $Q-p < 0.0001$), ($Q = 91.3336$; $I^2 = 93.43$; 95%CI: 88.91-96.11; $Q-p$

<0.0001) and ($Q = 51.2794$; $I^2 = 88.30$; 95%CI: 78.30 - 93.69; $Q-p < 0.0001$). However, for studies investigating *S. aureus*, moderate heterogeneity was observed ($Q = 13.5396$; $I^2 = 55.69$; 95%CI: 0.00 - 80.97; $Q-p < 0.0352$).

University campus / Residential houses: High heterogeneity was observed from the studies investigating three pathogens including *E. coli*, *S. aureus*, *P. aeruginosa* ($Q = 134.0283$; $I^2 = 95.52$; 95%CI: 92.86-97.19; $Q-p < 0.0001$), ($Q = 42.3304$; $I^2 = 85.83$; 95%CI: 72.83-92.61; $Q-p < 0.0001$) and ($Q = 31.5330$; $I^2 = 80.97$; 95%CI: 61.56 - 90.58; $Q-p < 0.0001$) respectively. For studies investigating *K. pneumoniae*, moderate heterogeneity was observed ($Q = 17.1543$; $I^2 = 65.02$; 95%CI: 21.35 - 84.45; $Q-p < 0.0087$).

Wastewater/ Broken sewage: High heterogeneity was observed from the studies investigating four pathogens including *E. coli*, *S. aureus*, *P. aeruginosa* and *E. faecium* ($Q = 35.1989$; $I^2 = 97.16$; 95%CI: 92.69-98.90; $Q-p < 0.0001$), ($Q = 22.6646$; $I^2 = 95.59$; 95%CI: 87.18 - 98.48; $Q-p < 0.0001$), ($Q = 14.9064$; $I^2 = 93.29$; 95%CI: 78.01 - 97.95; $P < 0.0001$) and ($Q = 13.7057$; $I^2 = 92.70$; 95%CI: 75.50 - 97.83; $Q-p < 0.0001$) respectively.

Nonspecific location: High heterogeneity was observed from the studies investigating two pathogens including *S. aureus* and *E. coli* ($Q = 38.1214$; $I^2 = 94.75$; 95%CI: 88.02 - 97.70; $Q-p < 0.0001$) and ($Q = 13.6833$; $I^2 = 85.38$; 95%CI: 57.08 - 95.02; $Q-p = 0.0011$).

3.3.3.5 Prevalence based on diagnostic methods

Two different diagnostic methods were used by the eligible studies to identify pathogens from houseflies, the combination of two diagnostic methods; culture and PCR had the highest heterogeneity in studies investigating *E. coli* and *P. aeruginosa* ($Q = 6303.3062$; $I^2 = 99.75$; 95%CI: 99.72 - 99.77; $Q-p < 0.0001$) and ($Q = 490.0658$; $I^2 = 96.74$; 95%CI: 95.76 - 97.48; $Q-p < 0.0001$). Lowest heterogeneity ($Q = 25.1695$; I^2

=36.43; 95%CI: 0.00 - 64.46 $Q-p = 0.0669$ was observed with the studies investigating *E. faecium* using both culture and PCR techniques Table 2 – 6).

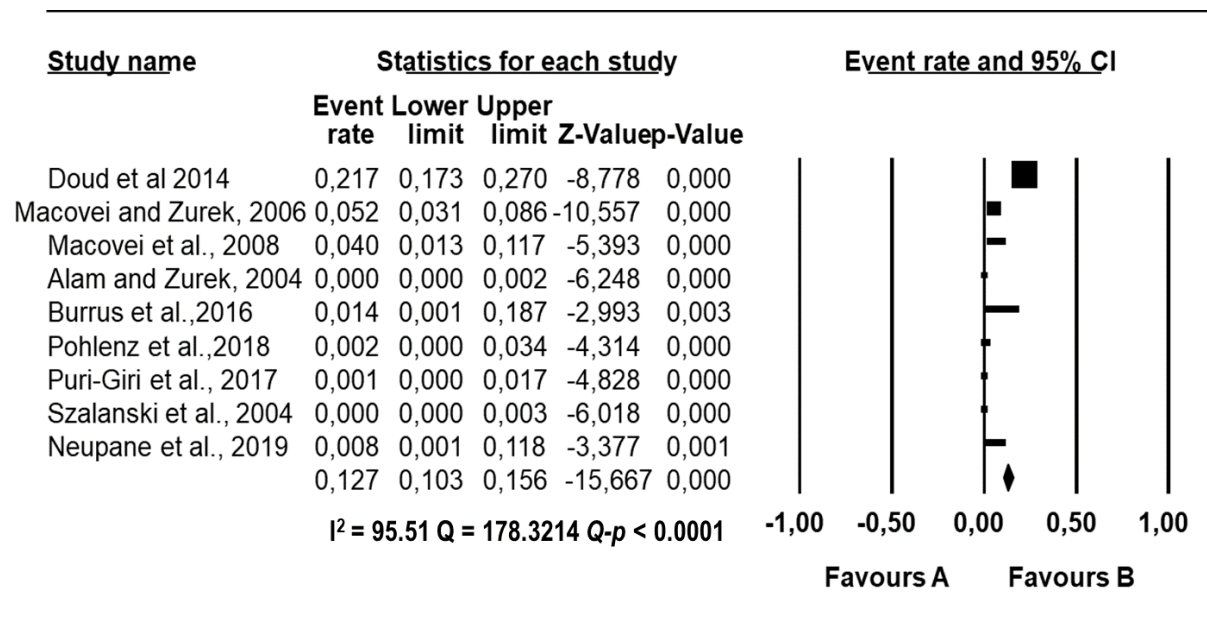


Figure 3.8 Forest plot of the point estimates owing to *E. faecium* from America. The squares demonstrate the individual point estimate. The diamond at the base indicates the pooled estimates from the total studies.

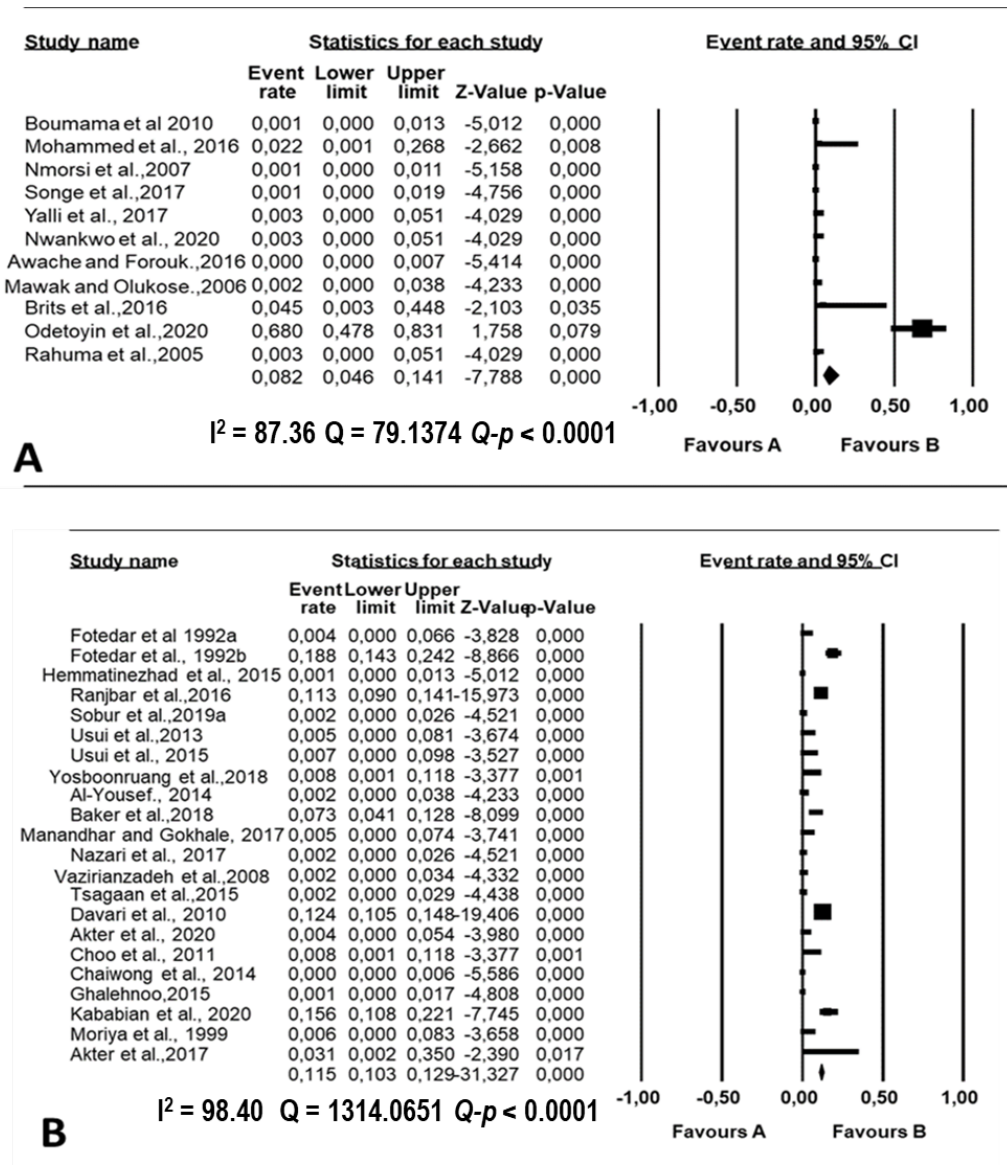


Figure 3.9 Forest plot of the point estimates owing to *K. pneumoniae* from (A) Africa and (B) Asia. The squares show the individual point estimate. The diamond at the base indicates the pooled estimates from the total studies.

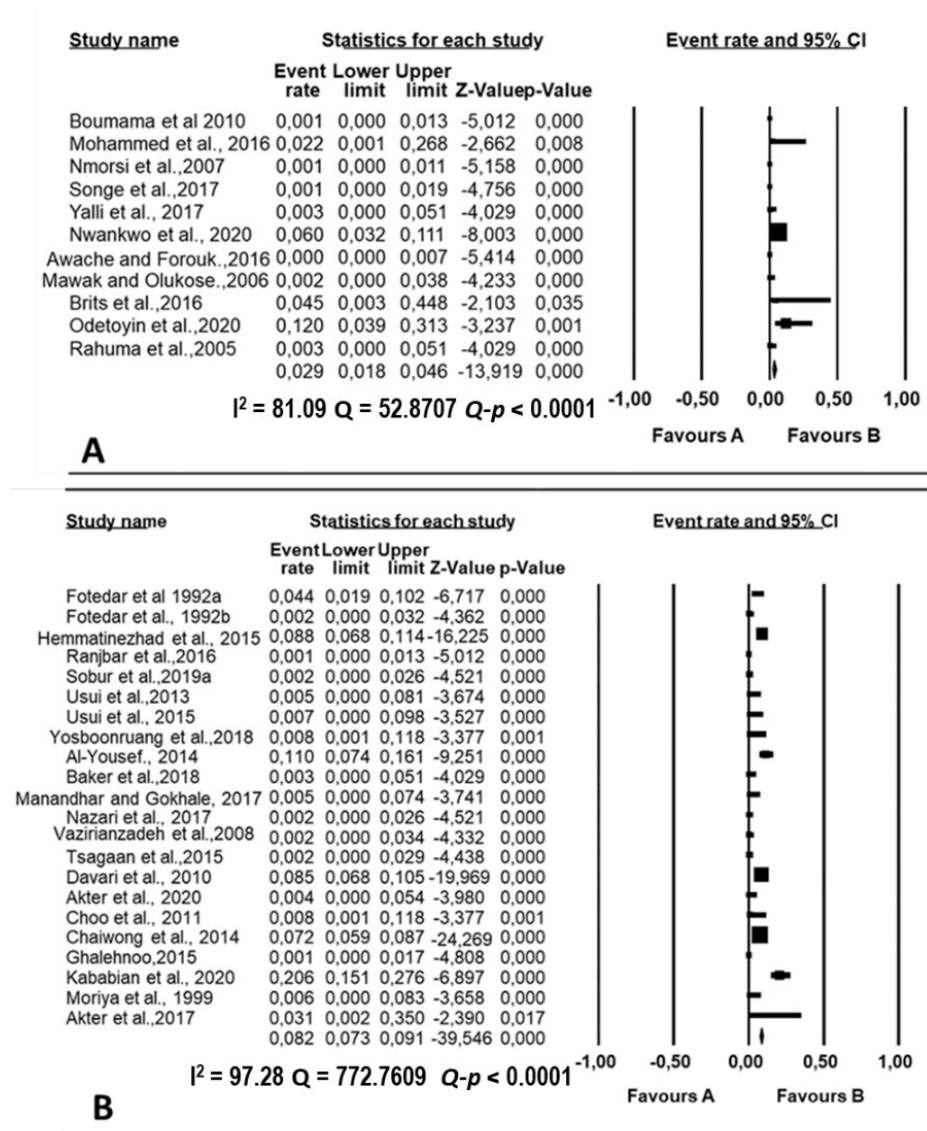


Figure 3.10 Forest plot of the point estimates owing to *P. aeruginosa* from (A) Africa and (B) Asia. The squares demonstrate the individual point estimate. The diamond at the base indicates the pooled estimates from the total studies.

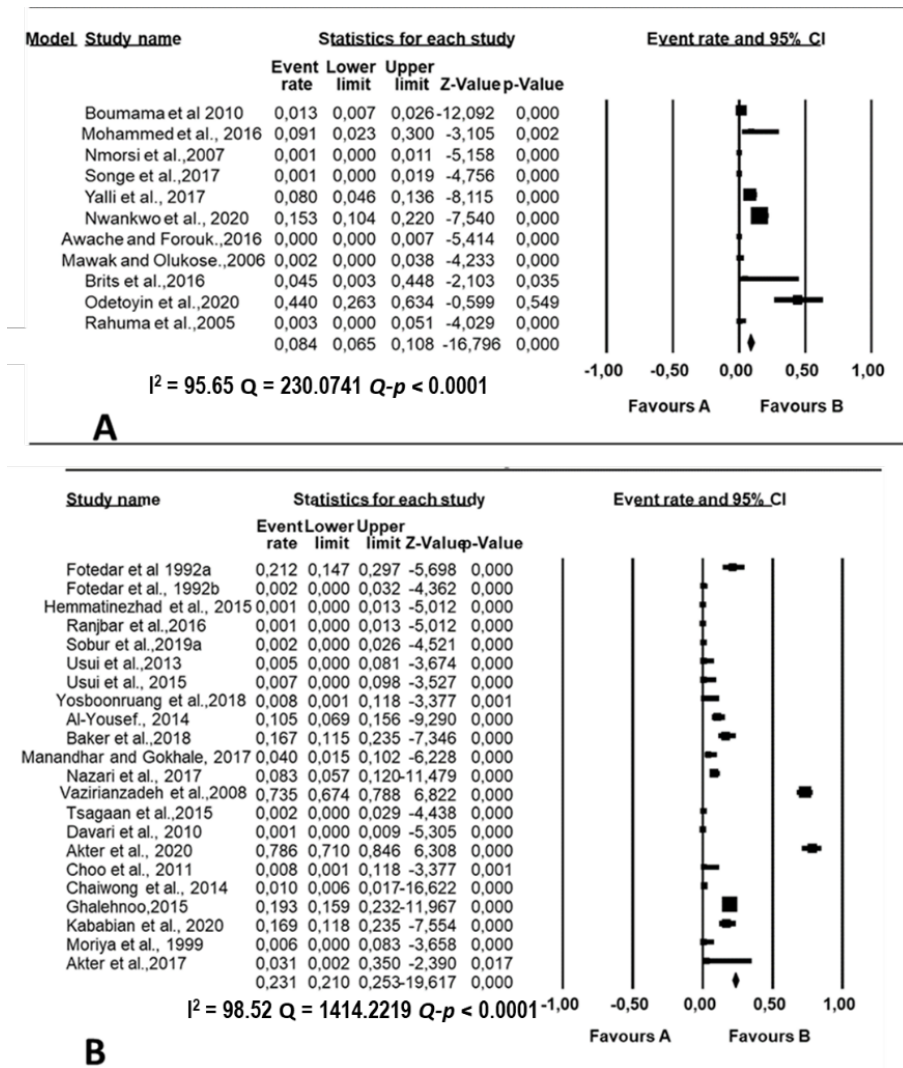


Figure 3.11 Forest plot of the point estimates owing to *S aureus* from (A) Africa and (B) Asia. The squares demonstrate the individual point estimate. The diamond at the base indicates the pooled estimates from the total studies.

3.3.3.6 Publication bias

According to the data observed from the funnel plot by virtual inspection, for all bacterial species namely, *E. coli*, *E. faecium*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*, no publication bias was observed as the funnel was symmetrical (Figure 4). Thus, there was significant asymmetry for the analysis of all the selected bacterial pathogens.

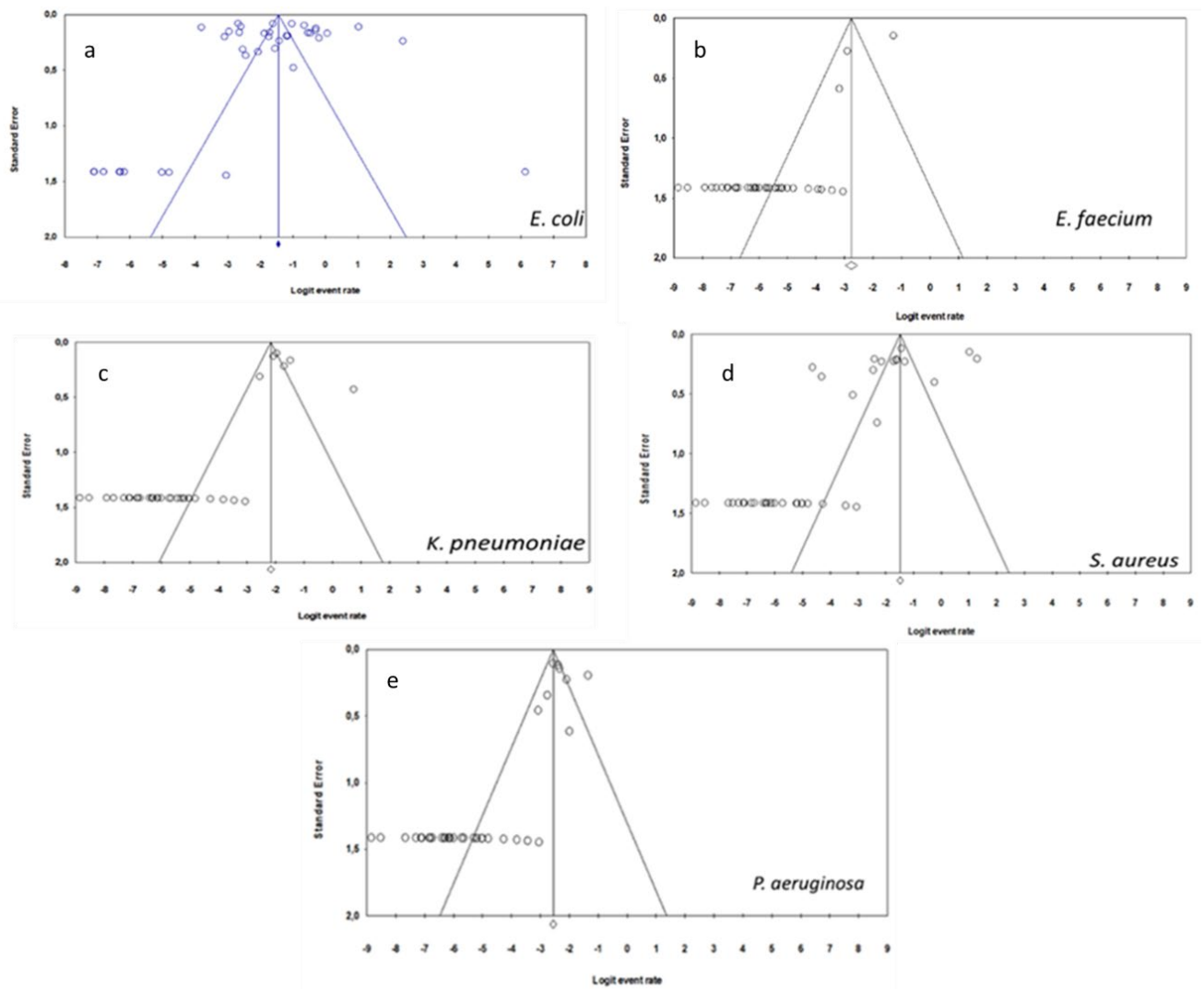


Figure 3.12 Funnel plots for studies investigating: (a). *E. coli* (b). *E. faecium* (c). *K. pneumoniae* (d) *S. aureus* and (e) *P. aeruginosa*.

3.4 DISCUSSION

This study undertook a systematic review and meta-analysis to estimate the pooled prevalence of the occurrence of pathogenic bacterial species of medical and veterinary importance carried by houseflies globally. The number of pathogens reported by the studies for qualitative synthesis varied greatly with most reporting on pathogenic and virulent bacterial strains. Thus, the pathogenic gram-negative bacteria were by far the most frequent with most studies as compared to Gram-positive bacteria. Different bacterial species found on houseflies depend on the location where the insects are captured (Cervelin *et al.*, 2018). This study has also proved that houseflies are in abundance in different settings and locations. These findings are in accordance with the review by Issa, (2019) where they indicated that houseflies live closely with humans and domestic animals and are often found in areas of human activities such as restaurants, hospitals, food centers, food markets, fish markets, and slaughterhouses. With the available information to date, there is evidence that houseflies are moderate to high in various human and animal settings harbouring clinically and veterinary relevant bacterial species in abundance (Gupta *et al.*, 2012).

Musca domestica is most frequently associated with zoonoses worldwide (Wales *et al.*, 2010). Throughout history, housefly colonization by bacteria has been reported previously by other studies including the study by Moorhead and Weisser (1946) where they recorded and proved the survival of *Staphylococcus* strains in the gut and excreta of houseflies. Additionally, other studies also documented that *M. domestica* is becoming heavily colonized by various bacterial genera including *Staphylococci*, *Streptococci*, and *Salmonellae* (Mawak and Okulose, 2006). Thus, houseflies have been implicated as vectors or transporters of various human pathogens, including *Vibrio cholerae*, Enterobacteriaceae pathogens, *Staphylococcus aureus*, and *Pseudomonas* spp. (Davari *et al.*, 2010; Ahmed *et al.*, 2013).

The most frequently reported bacterial species by various studies were: (i) *Escherichia coli*, the bacterium that causes nosocomial infections and bloody diarrhoea and haemorrhagic colitis in humans (Iwasa *et al.*, 1999; Wales *et al.*, 2010; Blaak *et al.*,

2013; Poudel *et al.*, 2019); (ii) *Enterococcus faecium*, the most important nosocomial pathogens of humans, causing urinary tract infections, bloodstream infections, endocarditis, and wound infections (Macovei *et al.*, 2008; Hammerum, 2012; Selleck *et al.*, 2019); (iii) *Klebsiella pneumoniae*, which cause infections of the respiratory tract and urinary tract, as well as post-operative wound infections (Blaak *et al.*, 2013; Ranjbar *et al.*, 2016); (iv) *Pseudomonas aeruginosa*, cause disease in humans and other animals (Banjo *et al.*, 2005); and (v) *Staphylococcus aureus* which is capable of causing human illness and food poisoning (Banjo *et al.*, 2005).

Antimicrobial resistance results in a serious public health problem, for it weakens the treatment efficacy against bacterial infections and this poses greater health risks (Wei *et al.*, 2013; Russell *et al.*, 2018). Some studies have reported some bacterial isolates which are resistant to significantly more than one of the commonly used antibiotics tested (Rahuma *et al.*, 2005; Hemmatinezhad *et al.*, 2015; Nazari *et al.*, 2017; Nwankwo *et al.*, 2019; Akter *et al.*, 2020; Odentoyin *et al.*, 2020). According to Russell *et al.*, (2018), dipterans that associate with livestock, livestock waste products and cadavers have the potential to acquire livestock-associated antibiotic-resistant bacteria and transmit them to humans. In the year 2017, the World Health Organization made a list of antibiotic-resistant priority pathogens which present a great threat to humans (WHO, 2017). Gram-negative bacterial pathogens including *E. coli*, *K. pneumoniae*, *P. aeruginosa* and Gram-positive *E. faecium* and *S. aureus* were the most listed under the critical and high categories concerning the urgent need of new antibiotics (WHO, 2017). Owing to the distinctive structure amongst the Gram-negative and Gram-Positive bacteria, the Gram-negative bacteria appear to be more resistant to antibiotics and cause significant morbidity and mortality worldwide (Breijyeh *et al.*, 2020).

The release of antibiotic-resistant bacteria expressing antibiotic resistance genes into the environment creates an opportunity for other insects to acquire livestock-associated antibiotic-resistant bacteria (Russell *et al.*, 2018). Some studies in this review have reported on the presence of resistance genes including mobile colistin, tetracycline and extended-spectrum β -lactamase gene, from bacterial isolates

identified from houseflies. This raises a very great concern since colistin is considered a last-resort antimicrobial for treating patients infected with multidrug-resistant Gram-negative bacteria (Zhang *et al.*, 2018). The spreading of the plasmid-encoded extended-spectrum β -lactamase gene which can confer resistance to third generation cephalosporins is the major problem because third generation cephalosporins are clinically important antimicrobials in human and veterinary medicine (Usui *et al.*, 2013). A review by Onwugamba *et al.*, (2018) on the role of flies in the spread of antimicrobial resistance also revealed that 'filth flies' are colonized by clinically important antimicrobial-resistant bacteria, including extended-spectrum beta-lactamase, carbapenemase-producing, and colistin-resistant bacteria. The presence of these resistant genes from the bacterial isolates simply indicates that houseflies can significantly disseminate bacteria carrying antibiotic-resistant genes from various environmental settings to humans (Zhang *et al.*, 2018).

In this study, we observed high variance in the number of studies conducted on bacterial pathogens harboured by houseflies per continent. Thus, Asia had the highest number of eligible studies followed by America, Africa, and Europe respectively. Many studies in Asia can be because, houseflies originate from Central Asia (Sanchez-Arroyo and Capinera, 1998). However, they are now common in all climates ranging from tropical regions to temperate climates (Sanchez-Arroyo and Capinera, 1998; Khamesipour *et al.*, 2018). Hence there's many studies observed from other continents including America (Olsen and Hammack., 2000; Macovei and Zurek, 2006; Macovei *et al.*, 2008; Burrus *et al.*, 2016; Alves *et al.*, 2018; Poude *et al.*, 2019, Africa (Brits *et al.*, 2016; Songe *et al.*, 2017; Ibrahim *et al.*, 2018; Nwankwo *et al.*, 2020) and Europe (Rosef *et al.*, 1983; Blaak *et al.*, 2014; Davies *et al.*, 2014; Solà-Ginés *et al.*, 2015).

Culturing and plating conventional microbiological methods are still regarded as a gold standard, as they enable the identification of various bacterial species effectively (Kotsilkov *et al.*, 2015). However, the combination of both microbiological culturing and molecular methods is increasingly adopted (Langley *et al.*, 2015). Due to the use of different pathogen detection methods (PCR and culture including selective media

and/or broth enrichment), the bacterial carrier rates from flies must be cautiously compared (Onwugamba *et al.*, 2018). Most of the studies in this review used selective culture media to identify predominantly pathogenic bacteria or specific species. However, medium preferences and growth condition optima of individual bacterial species may influence the observation of species diversity and the abundance of individual bacteria isolated (Brits *et al.*, 2016). The combination of both culture and molecular techniques has been shown to improve the identification and isolation rate of bacterial species. However, advanced methods including PCR are costly (Khamesipour *et al.*, 2018). A review by Adzitey *et al.*, (2013), showed that studies using molecular methods (PCR and/or sequences) yield more pathogens compared to studies using standard cultural methods. However, Gupta *et al.*, (2012) showed that there are benefits and limitations concerning the use of either approach for diagnosis.

Notably, all studies included in this systematic review and meta-analysis were conducted differently, as some studies investigated many samples while others investigated different settings and utilizing various diagnostic techniques. These parameters resulted in the high heterogeneity observed in this study. According to Oppong *et al.*, (2020), heterogeneity could partially be attributed to the large variation in sampling and diagnostic techniques applied for the detection of bacterial pathogens. This is because different techniques differ in their detection sensitivity, specificity, as well as positive and negative predictive values. There was no statistical significance at the study level, but we observed statistical significance at the meta-analysis. Substantial heterogeneity was observed in studies included in our analyses which can largely be attributed to variation in sample size, sampling method and analysis of data. Some studies presented a small sample size resulting in effect estimates with wide confidence intervals.

This study has a considerable number of strengths including (i) this study summarizes currently existing knowledge and helps in identifying areas that lack adequate evidence, thereby producing new research questions. (ii) the overall consistency was checked for the searching process, study choice and inclusion/exclusion criteria. All the duplicated records were removed. The authors independently examined the titles,

abstracts and full-text articles. This was done to negate any possibility of errors. (iii) the application of a consistent reviewing approach and extracting relevant data from the available published studies allowed us to reliably determine the pathogenic bacteria isolated from houseflies by various studies around the world. And, how frequent these studies report on the antibiotic susceptibility profiles and resistant genes in common bacterial pathogens in different countries (iv) exclusion of duplicate studies and studies that are not specific with genus and species of the fly, studies using laboratory-bred houseflies while including only studies with genus and species confirmed (v) and considering studies with samples collected from different human and animal settings.

Several limitations have been identified which included the following, (i) only journal papers published in the English language were considered. This was due to lacking resources for the translation of papers that might have been published in other languages. (ii) Few studies have failed to describe the sample size despite the importance of considering the sample size in all investigational studies. Sample size calculation is ethically important and forms part of the early stages of leading epidemiological, clinical, or laboratory studies (Faber and Fonseca, 2014). (iii) Furthermore, some countries had more research reports compared to others.

In conclusion, this systematic review and meta-analysis have provided a global overview on the carriage of significant bacterial pathogens including antibiotic-resistant bacteria identified in houseflies from different regions in the world. Houseflies are in abundance and found almost in every human and animal habitation and this poses a serious threat to public health. Most of the studies in this review predominantly identified pathogenic bacteria including *E. coli*, *E. faecium*, *K. pneumoniae*, *P. aeruginosa* and *S. aureus*. Few studies presented data on antibiotic susceptibility profile and resistant genes from housefly and this is of great concern, as flies have the potential to rapidly and widely disseminate antimicrobial-resistant genes. Studies in this review were found to employ different diagnostic methods including culture and PCR or the combination of both techniques. Further studies with a special focus on antibiotic susceptibility profiles and resistant genes from bacteria identified

from houseflies will provide more insight into a global threat to human and animal health (Zhang *et al.*, 2018). Future systematic reviews may also include the following: (i) data from different life cycles of housefly including eggs, larvae and pupa (ii) data from culture-independent studies that allow the identification of the cultivable and non-culturable fractions of bacteria present in houseflies. The findings of this study provide more insight into the carriage of significant bacterial pathogens of distinct taxonomic identities by *Musca domestica* from different geographical locations across the world. A continuous investigation is required to completely comprehend the significance of medically and veterinary important pathogenic bacteria harboured by houseflies. In addition, further investigations are needed to quantify the contamination level to analyse the risk of bacterial contamination and dissemination by houseflies.

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CHAPTER 4

CHARACTERIZATION OF HOUSEFLIES (DIPTERA: MUSCIDAE) FROM HOSPICES IN BLOEMFONTEIN AND JOHANNESBURG, SOUTH AFRICA

4.1 INTRODUCTION

Insects are amongst the most diverse and ecologically significant organisms on earth. Despite this, their diversity and abundance are however, rarely quantified (Siti *et al.*, 2017), particularly in human surroundings. Accurate species identification and up-to-date locality information are basics for many fields in biology including animal and human health (Galan *et al.*, 2012; Xiong *et al.*, 2012). Therefore, clinical entomology is essential for the effective application of improving the health care delivery system to reduce the prevalence of human infections and diseases.

Muscidae is the most abundant insect family in close association with humans and occupies various environmental niches. The environments include open markets, funfairs, restaurants, garbage dumps, animal pens, and confined animal feeding operations (Abbas *et al.*, 2013; Almeida and Giuffrida, 2014). Accurate identification of muscoid flies concerning the housefly (*Musca domestica*) is an essential factor for both epidemiological studies and the precise formulation of vector control strategies (Bhakdeenuan *et al.*, 2012).

Current techniques for Muscidae identification and classification involve the use of published identification keys and microscopy. This is applied based on the specimen's morphological features that include the shape, colour and size of different parts of an adult fly (Catts and Goff., 1992; Xiong *et al.*, 2012). Diverse structural features on the thorax, leg chaetotaxy and wing venation can only be witnessed and notable in the mature stage of Muscidae members. According to Grzywacz *et al.*, (2017), the uses of geometric morphometric analysis of wings allow the detection of subtle differences between specimens on various taxonomic levels. However, morphological identification can be complicated and sometimes impossible due to similarities among

species, especially in immature stages and on adult specimens as well (Yusseff-Vanegas, 2017). Additionally, morphological identification of muscoid species requires a significant grade of skill and taxonomic expertise (Hebert *et al.*, 2003; Contreras-Gutierrez *et al.*, 2014).

Due to challenges presented by morphological identification, DNA barcoding was developed (Hebert *et al.*, 2003). This initiative has been appreciated by scientists globally due to the connectivity and common language of DNA sequences, which allows for the advancement in taxonomy and systematic studies of various groups of organisms, including insect vectors (Contreras-Gutierrez *et al.*, 2014). These advanced molecular techniques are therefore proposed to overcome the challenges in species identification because of limitations shown by the traditional morphology-based taxonomic system (Galan *et al.*, 2012; Ren *et al.*, 2018).

The molecular barcoding technique combines the use of DNA markers and is applied to identify species by short standardized sequences of DNA. This short-standardized sequence of DNA displays a significant amount of sequence variation among species and conserved sequences within the species (Herbert, *et al.*, 2003; Galan *et al.*, 2012). The system corresponds to the Barcode of Life Project, which proposes a single universal system to identify all organisms at the species level (Xiong *et al.*, 2012; Galan *et al.*, 2012).

The use of specific DNA primers targeting specific sequences in the DNA of an organism has enabled scientists who are not trained as taxonomists to identify muscoid and other species rapidly and accurately (Galan *et al.*, 2012). This technique can be applied to all developmental life stages of a muscoid species and on ancient, incomplete and damaged samples (Boehme *et al.*, 2012; Galan *et al.*, 2012). Thus, when anatomical samples are destroyed or damaged, molecular barcoding grants an advantage of using a portion of the specimen or a non-invasive sample for accurate species identification (Boehme *et al.*, 2012; Galan *et al.*, 2012).

DNA-based identification method has been applied using the cytochrome oxidase 1 (CO1) gene. This region is significant for enabling inventories of biodiversity and performing species identifications (Contreras-Gutierrez *et al.*, 2014). The CO1 gene

has been applied broadly for species identification due to its high degree of nucleotide variation (Boehme *et al.*, 2012). Furthermore, this gene region is suitable for this role because its mutation rate is often rapid enough to differentiate between closely related species. Additionally, this gene is conserved amongst conspecifics (Hebert *et al.*, 2003). The *CO1* gene has been demonstrated to be suitable for clear differentiation and identification in forensically relevant Diptera and has mostly become the standard in DNA barcoding for animal species (Boehme *et al.*, 2012). Lastly, it has been widely utilized for animal molecular identification to accomplish correct identification of an unknown sample (Ren *et al.*, 2018; Rotty *et al.*, 2018).

However, for accurate identification of species, GilArriortua *et al.*, (2014) have recommended a second nuclear genetic marker which is referred to as the internal transcribed spacer 2 (*ITS2*) as an additional gene region in barcoding locus for taxonomic species determinations in calliphorids. This gene region is a non-coding nuclear ribosomal DNA and it is situated between the small-subunit ribosomal RNA (*rRNA*) and large-subunit *rRNA* genes in the chromosome or the corresponding transcribed region in the polycistronic *rRNA* precursor transcript (Fromm *et al.*, 2017). This non-coding gene region has been identified as an alternative to *CO1*, especially when *CO1* does not possess the capability to differentiate amongst the species (Nelson *et al.*, 2008). However, one of the limitations of this gene is that it cannot be used to differentiate between geographical populations.

The Muscidae family could be distinguished through the variation in *16S rRNA* sequence (Li *et al.*, 2010). The use of *16S rRNA* gene is believed to be effective as a morphological method in the identification of Muscidae species (Li *et al.*, 2010). In addition, the most recent study conducted by Grzywacz *et al.* (2021), utilized the multilocus Sanger sequencing, anchored hybrid enrichment and restriction-site associated DNA sequencing approaches to examine relationships within Muscidae as well as their classification.

In the South African context, hospices are places that house terminally ill patients suffering from diseases such as cancer. Additionally, they are used to care for acquired immune deficiency syndrome (AIDS) patients since the year 1980 (Gwyther and Rawlinson 2007; Makhele and Mulaudzi, 2012). Thus, as non-governmental

organizations, hospices provide active total care of patients whose disease is non-reactive to treatment. The main goal is to maintain the good quality of life for patients and their families (WHO, 2014).

The current study aimed to determine the occurrence and classify the houseflies in hospices in Bloemfontein and Johannesburg, South Africa. The study further sought to determine the phylogenetic relationship of these houseflies (*Musca* spp.) in comparison to other related muscoid species that are presented globally. To achieve this, the following objectives were formulated:

- (i) To determine the occurrence of houseflies and identify them using morphological and genetical analysis
- (ii) To conduct phylogenetic analysis of housefly's *16S rRNA*, *CO1* and *ITS2* genes using MEGAX software.

4.2 MATERIALS AND METHODS

4.2.1 Study areas

This study focused on two of the nine provinces of South Africa namely, Gauteng and Free State Provinces (Figure 4.1) for the collection of housefly samples. The entire landscape size, seasonal climatic conditions including vegetation from two sampled provinces varies significantly as documented by Mucina and Rutherford (2006).

4.2.1.1 Lebone Village and Sunflower house in Free State Province

The housefly specimens were collected at two hospices, namely, Lebone Village (29°03'51.7"S 26°20'29.4"E) and Sunflower House (29°07'36.7"S 26°12'23.1"E) in Bloemfontein, Free State (Figure 4.1). The Free State Province is the third largest province in South Africa in terms of land area, with approximately 129,825 km². This province experiences an average of 500-600 mm summer-rainfall throughout November to April (summer to autumn) in the following year, with average summer temperatures of 15°C - 32°C (Mucina and Rutherford, 2006). However, the province can be extremely cold during the winter seasonal months (i.e., May to October) with

average winter temperatures of 1°C - 17°C mostly in the eastern mountainous regions. The western and southern regions of the Free State Province are semi-desert (Mucina and Rutherford, 2006; Dingaan *et al.*, 2013).

4.2.1.2 Sparrow Ministries of Help in Gauteng Province

Housefly specimens were also collected at Sparrow Ministries of Help (26°05'34.2"S 27°59'10.6"E) situated near the city of Johannesburg, Gauteng Province (Figure 4.1). Gauteng lies on the great interior plateau of South Africa and it is the smallest province in the country, yet, is among the provinces with the largest population sizes. The province's climate is warm and temperate with the average annual rainfall of 650 mm. The climate in Johannesburg, which is one of the cities of Gauteng Province, is never humid and uncomfortably hot. The province experienced a minimum average temperature of 17°C with a maximum of 28°C during summer seasons and the minimum average temperature of 0°C with the maximum of 20°C during winter seasons, the area experiences the coldest weather during mid-winter (July and August) (www.south-africa-info.co.za; Mucina and Rutherford, 2006).

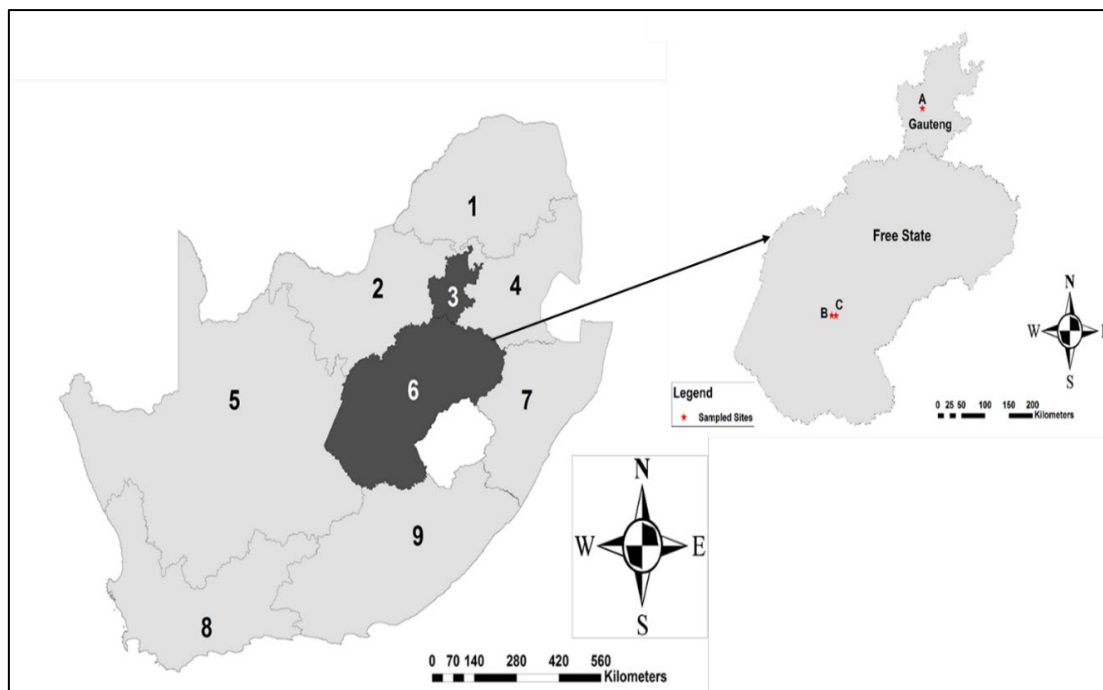


Figure 4.1 Map of South Africa showing the two sampled provinces (3 and 6) out of the 9 provinces. (A) Sparrow Ministry of Help hospice in Johannesburg, Gauteng Province at number 3 and (B) Lebone Village orphanage and (C) Sunflower House in Bloemfontein, Free State Province at number 6.

4.2.2 Housefly sampling

Houseflies were collected between September 2019 and February 2020 from three targeted registered hospices as indicated in Figure 4.1. In all sampled hospices, a minimum of three sticky traps were set for at least three to five days for a period of two weeks in the kitchens and dining halls to capture houseflies that were near areas where food was either prepared or consumed (Plate 4.1-4.3). The sticky traps were monitored daily.

The captured houseflies were immediately stored in a properly labeled container with 70% ethanol and transported in an icebox to one of the molecular biology laboratories in the North-West University, Potchefstroom Campus. Afterwards, the collected houseflies were stored in -40°C until further processing. Upon arrival at the university laboratory, all sampled specimens from different hospices were allocated into individual sterile vials handled with pair of sterile forceps. The flies were identified under a light microscope according to their morphology and recorded. Subsequently, all identified housefly specimens were separated from the remaining by-catch samples.



Plate 4.1. Sticky traps used to capture houseflies at Lebone Village of Orphanage. (A) Dinning area, (B and C) Food preparation area/ kitchen.

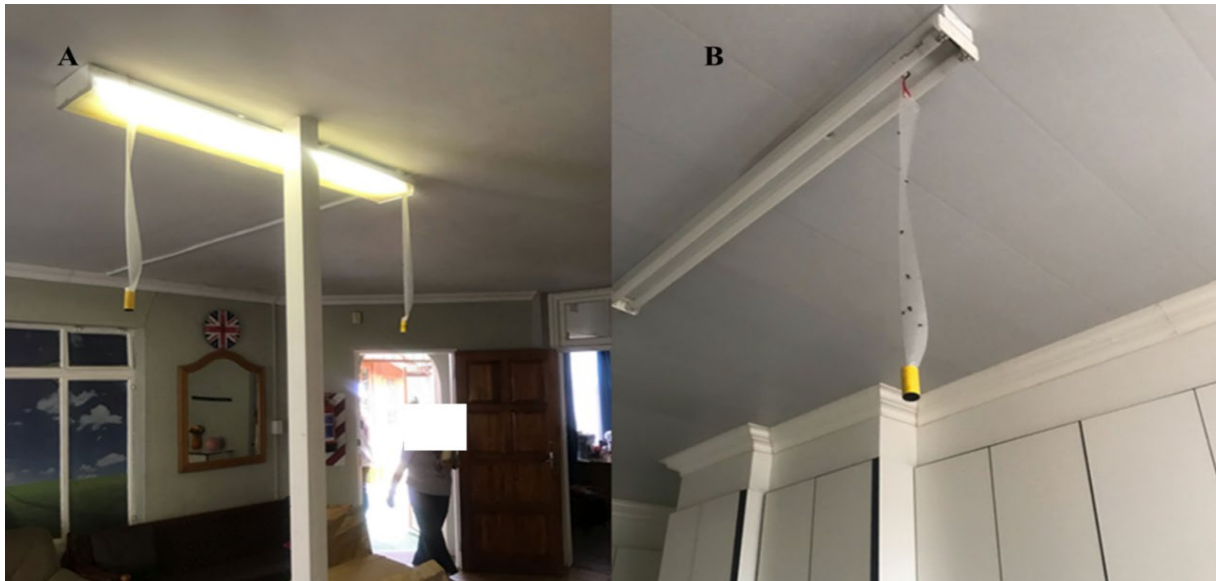


Plate 4.2. Sticky traps used to capture houseflies at Sunflower House. (A) Dining area, (B) Food preparation area/ kitchen.



Plate 4.3. Sticky traps used to capture houseflies at Sparrow Ministry of Help. (A and B) Food preparation area/ kitchen.

4.2.3 Morphological characterization of houseflies

Prior to morphological identification, houseflies were washed with glue remover (Insects Science, South Africa) to free specimens of any glue residues acquired from the sticky traps. Glue-free flies were then identified using Wild Heerbrugg M5 stereo

microscope (Heerbrugg, Switzerland) with reference to literature of Service, (2012), Al-Shareef, (2016) and Sawaby *et al.* (2018). The Nikon AZ 100 M zoom microscope (Nikon Inc., Tokyo, Japan) was used to capture sequential photographs of different features of the houseflies. To identify houseflies collected from hospices, species identification keys were used to confirm the observed features from the captured specimens. All the flies were morphologically identified and grouped according to hospice.

4.2.4 Molecular characterization of the flies

4.2.4.1 DNA extraction from houseflies

Housefly specimens were washed twice with 70% ethanol and once with double distilled water (ddH₂O) to remove any contaminants before DNA extraction. Subsequently, captured flies were pooled (five flies per pool) to increase the DNA yield and then crushed into sterile 1.5 ml Eppendorf tubes and subjected to DNA extraction using Qiagen DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. Housefly specimens were then homogenized in a PowerBead tube containing 60 µl of solution C1 the mixture was vortexed for 10 minutes. The mixture was then centrifuged at 10 000 x *g* for 30 seconds. After the centrifugation process was complete, the supernatant was transferred into a clean 2 ml collection tube and 250 µl of solution C2 was added into the supernatant and the mixture was vortexed for 5 seconds and incubated at 8°C for 5 minutes then centrifuged for 1 minute at 10 000 x *g*. Avoiding the pellet, 600 µl of supernatant was transferred into a clean 2 ml collection tube, then 200 µl solution C3 was added and vortexed and incubated at 8°C for 5 minutes. The tube was then centrifuged for 1 minute at 10 000 x *g*. Avoiding the pellet again, approximately 750 µl of the supernatant was transferred into clean 2 ml collection tubes, solution C4 was then mixed and the amount of 1200 µl was added to the supernatant and vortexed for 5 seconds. Thereafter, 675 µl of the mixture was added onto an MB spin column and centrifuged at 10 000 x *g* for a minute and the flow-through was discarded; this step was repeated twice until the entire sample has been processed. A total of 500 µl of solution C5 was added, centrifuged for 30 seconds at 10 000 x *g*, then the flow-through was discarded and centrifuged again for a minute at 10 000 x *g*. the MB spin column

was carefully placed into a clean 2 ml collection tube to avoid splashing solution C5 onto the column. A total of 100 µl from solution C6 was added to the center of the white filter membrane to elute the extracted DNA and centrifuged at room temperature for 30 seconds at 10 000 x g. Subsequently, the MB spin column was discarded and the DNA concentration and quality measured. Extraction yield and the quality of the DNA were verified by 1% agarose gel electrophoresis. Thereafter, the DNA samples were stored at -20°C, until further use.

4.2.4.2 Evaluation of the quantity and quality of isolated DNA

The quantity of DNA extracted from the samples was determined by spectrophotometry with a NanoDrop ND-1000 system (NanoDrop Technologies, Inc., Wilmington, DE, USA). The purity of DNA was determined spectrophotometrically from the ratio of absorbance at 260 and 280 nm (A₂₆₀/A₂₈₀).

4.2.5 PCR amplification of 16S *rRNA*, cytochrome oxidase 1 (*CO1*) and internal transcribed spacer 2 (*ITS2*) genes

Polymerase chain reaction (PCR) targeting the 16S *rRNA*, *CO1* and *ITS2* genes were conducted to identify houseflies to species level as well as to supplement morphological observations. The primer sequences shown in table 4.1 were synthesized by Inqaba Biotechnical Industries (Pty) Ltd, Pretoria, South Africa and were used for various PCR reactions. All PCR reactions were performed with a final reaction volume of 25 µl. The final reaction mixture for both genes consisted of 1 µl of template DNA, 8.5 µl ddH₂O, 2X master mix with standard buffer (OneTaq Quick load buffer, 4 mM MgCl₂, 0.4 mM of each dNTP and 1 unit/µl of thermostable *Taq* polymerase (Thermo Scientific, USA), the primer mix contained 1 µM of each oligonucleotide primer. Genomic DNA of confirmed housefly in this study was used as a positive control and ddH₂O as a negative control.

To amplify the 555 bp product of 16S *rRNA*: PCR was initiated by denaturation at 95°C for 10 min, followed by 35 cycles of 95°C for 30 seconds, annealing at 55°C for 30 seconds, elongation at 72°C for 30 seconds and final elongation at 72°C for 1 minute.

To amplify the 710 bp product of *CO1*, the PCR conditions were set as follows: initial denaturation at 94°C for 5 min, followed by 35 cycles of the amplification steps including 94°C for 60 sec, 47°C for 60 sec and 72°C for 45 sec and finally extension at 72°C for 10 min.

To amplify the 418 bp product of *ITS2*: PCR was initiated by denaturation at 94°C for 5 min, followed by 35 cycles of 1 min at 94°C for template denaturation, 1 min at 52°C for primer annealing, and 1 min at 72°C for primer extension. The reaction was terminated by a 10 min elongation cycle at 72°C.

Following the amplification, 5 µl amplicon was resolved by gel electrophoresis using 1% agarose gel stained with ethidium bromide and visualized under UV light.

Table 4.1 Primer sequences for amplification of 16S rRNA, CO1 and ITS2 regions

Region to amplified	Primer Name	Sequence (5'-3')	Amplification length (bp)	Reference
16S rRNA	LR-J-12887	CCGGTCTGAACTCAGATCACGT	555	Guo <i>et al.</i> , 2012
	LR-N-13398	CGCCTGTTTAACAAAAACAT		
CO1	LCO1490_F	GGTCAACAAATCATAAAGATATTGG	710	Rotty <i>et al.</i> , 2018
	HCO2198_R	TAACTTCAGGGTGACCAAAAAATCA		
ITS2	ITS2_F	TGCTTGGACTACATATGGTTGA	418	Song <i>et al</i> 2008
	ITS2_R	GTAGTCCCATATGAGTTGAGGTT		

4.2.6 Purification of amplicons and sequencing

Twenty microliters of positively amplified PCR products were sent to a commercial sequencing facility, at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa for purification and sequencing in both directions. The quality of resultant sequences was assessed using both MEGA X (Kumar *et al.*, 2018) and Geneious Ver. 7.1 [<http://www.geneious.com>] (Kearse *et al.*, 2012). This was done before consensus sequences were generated from both forward and reverse sequence reads for 16S rRNA, CO1 and ITS2 fragments, respectively.

4.2.7 Phylogenetic analysis

Generated consensus sequences were edited using MEGAX (Kumar *et al.*, 2018). All edited *16S rRNA*, *CO1* and *ITS2* gene sequences were subsequently subjected to the Basic Local Alignment Search Tool for nucleotides (BLASTn) (www.ncbi.nlm.nih.gov/blast/) to confirm their identities. Sequences that belonged to a specific species were identified based on being the first and closest match in GenBank, with the identity match score of 90 up to 100% coverage. Gene sequences of species closely related to houseflies from the BLASTn in search results were subsequently downloaded from the NCBI database to be included in the phylogenetic analysis.

To determine the phylogenetic relationship of the houseflies collected from the three hospices (Lebone, Sparrow and Sunflower hospice) obtained from *16S rRNA*, *CO1* and *ITS2* gene sequences along with conspecific sequences downloaded from the NCBI database, all sequences representing various respective gene regions were aligned by ClustalW with default parameters on MEGAX for multiple and pairwise sequence alignments (Kumar *et al.*, 2018). MEGAX was used to subsequently edit and trim as well as to determine the appropriate evolutionary model to construct the maximum likelihood (ML) trees for the three amplified gene regions.

For the *16S rRNA* gene region, the best model selected was the Tamura 3-parameter model with 1000 bootstrap replicates to evaluate the tree topology (Nei and Kumar, 2000; Kumar *et al.*, 2018). There was a total of 13 *16S rRNA* gene sequences which were used to construct the tree whereby *Stomoxys calcitrans* (GenBank accession no: HM245739 and HM245747) were used as outgroups.

For the *CO1* gene fragment, the best model selected was the General Time Reversible with Gamma Distributed (G) rates (+G, parameter = 0.2962) with 1000 bootstrap replicates to evaluate the tree topology (Nei and Kumar, 2000; Kumar *et al.*, 2018). There was a total of 19 *CO1* gene sequences which were used to construct the tree whereby *Stomoxys calcitrans* (GenBank accession no: HQ945124 and GU804070) were used as outgroups.

For the *ITS2* gene region, the best model selected was the Tamura 3-parameter with Gamma Distributed (G) rates among sites set at 5.00 (+G, parameter = 0.0500) with 1000 bootstrap replicates to evaluate the tree topology (Kumar *et al.*, 2018; Nei and Kumar, 2000). There was a total of 23 *ITS2* gene sequences which were used to construct the tree whereby *Haematobia titillans* and *Stomoxys calcitrans* (GenBank accession no: EU376396 and HM185467) were used as outgroups.

All sequences produced from this study were deposited to the NCBI GenBank database under the following accession numbers: for *16S rRNA*- MZ702642, MZ702643, MZ702644, *CO1* -MW579487, MW579488, MW579489, MW579490, MW579491, & MW579492 and *ITS2* -MW584801, MW584802, , MW584803, , MW584804, , MW584805, MW584806, , MW584807, , MW584808, , MW584809 & MW584810, respectively.

4.2.8 Statistical analysis

The apparent density of fly population was calculated by dividing the number of flies caught by the number of traps deployed and the number of days of deployment and expressed as fly /trap/ day (FTD). The number of houseflies captured from the hospices in Bloemfontein and Johannesburg were captured. Error bars using descriptive statistics at 95% confidence intervals were calculated to accommodate the margin of error during the sampling process.

4.2.9 Ethical Approval

Prior to the commencement of the study, the research proposal was approved by the Biodiversity and Conservation Scientific Committee of the Unit for Environmental sciences and management, North-West University (NWU) and the Faculty of Natural and Agricultural Sciences Ethics Committee of the NWU (NWU-01760-20-A9).

4.3 RESULTS

4.3.1 Morphological analysis

A total of 657 houseflies were collected from the three sampled hospices (Table 4.2 and Figure 4.2). A summary representing other insects that were caught as by-catch is given in table 4.3. Identification of housefly species was based on their morphological characteristics based on previously published identification keys as follows: An adult housefly is medium sized (6 to 14 mm) in body length (Plate 4.4 A). The dorsal surface of the thorax has four broad black longitudinal stripes [Plate 4.4 B] (Service, 2012). The mouthparts are specifically adapted for sucking up fluid or semifluid foods. When not in use they are partially withdrawn into the head capsule (Plate 4.4 C) but are extended downwards in a telescopic fashion when the fly feeds. The most important identification character which contributed to distinguishing *Musca* species from other similar flies, is that the wing forth vein is bending up sharply to join the costa close to the third vein (Plate 4.4 D) (Service, 2012; Alikhan *et al.*, 2016). The abdomen is yellowish with a dark midline and somewhat irregular dark markings on the sides [Plate 4.4 E] (Service, 2012; Sawaby *et al.*, 2018).

Table 4.2 Number of flies caught by Sticky- traps and their apparent density in three hospices

Hospice	No of Sticky traps	Total number of <i>Musca domestica</i> (%)	AD	Insects captured as by-catch			
				Total number of * <i>Lucilia cuprina</i> (%)	AD	Total number of <i>Lucilia sericata</i> (%)	AD
Lebone Village	16	306 (46.6)	1.37	48 (14.1)	0.21	18 (11.6)	0.08
Sunflower House	8	93 (14.2)	0.83	28 (8.2)	0.25	14 (9.0)	0.13
Sparrow Ministries	12	258 (39.3)	1.54	265 (77.7)	1.57	123 (79.4)	0.73
Total	36	657	1.30	341	0.68	155	0.31

AD= Apparent density. * Photos of *Lucilia cuprina* and *L. sericata* are shown in appendix 2.

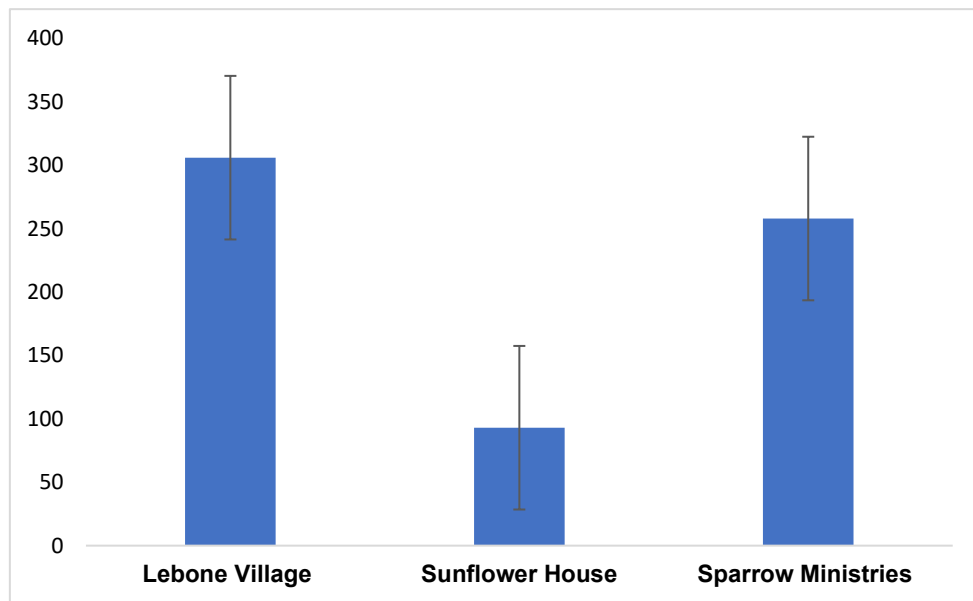


Figure 4.2 The number of houseflies (*Musca domestica*) captured from the three sampled hospices.

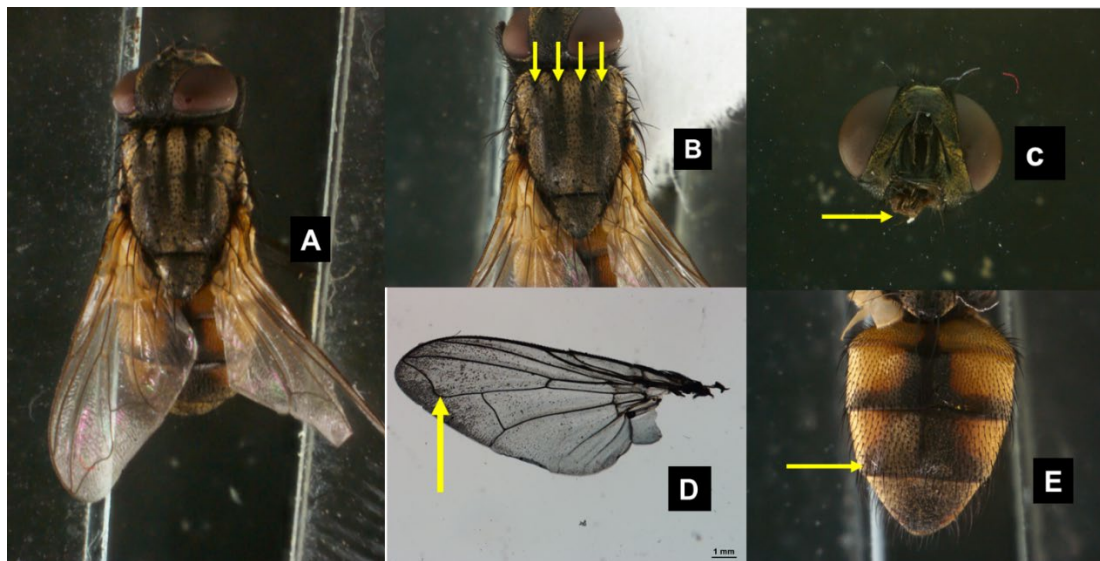


Plate 4.4. Morphological features of the housefly. (A) The dorsal view of the whole fly; (B) the dorsal features of the thorax with the arrows highlighting the four broad black longitudinal stripes (C) Dorsal view of the head with the arrow highlighting the proboscis (D) wing with the arrow indicating the 4th vein bending up sharply to join the costa close to 3rd vein. (E) Dorsal view of the abdomen with an arrow highlighting the position of the checked pattern.

4.3.2 Amplification of the fly DNA

A ratio of between 1.7 and 2 indicates the excellent quality of DNA (Rotty *et al.*, 2018). The *16S rRNA*, *CO1* and *ITS2* genes were successfully amplified from housefly DNA samples. The length of *16S rRNA*, *CO1* and *ITS2* sequences were 555 bp, 710 bp and 418 bp as shown from the electrophoresis gel images from Figure 4.2, 4.3 and 4.4, respectively.

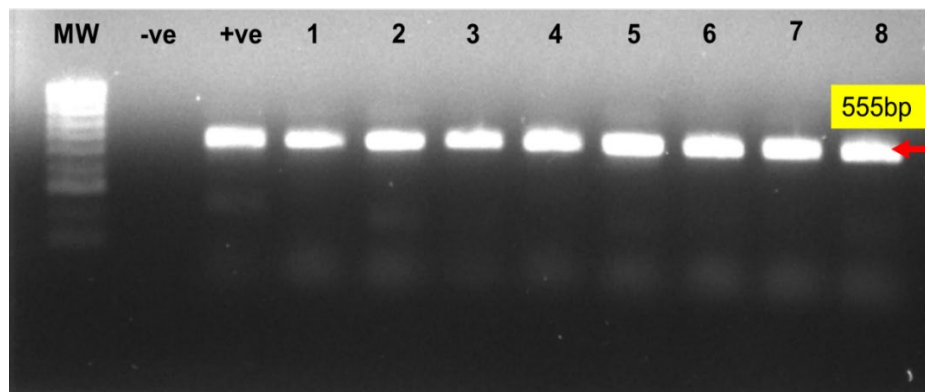


Figure 4.3 PCR amplification of *16S rRNA* gene. Lane M: Molecular weight marker (1kb); Lane -ve: negative control, +ve Positive control, Lane 1,2,3,4,5,6,7 and 8 are test samples from the sampled hospices for the amplification of the *16S rRNA* gene fragment.

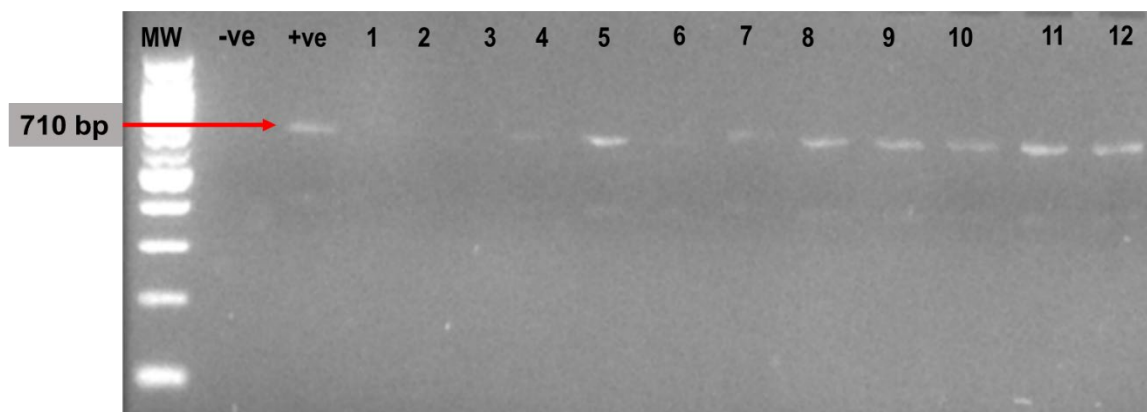


Figure 4.4 PCR amplification of *CO1* gene. Lane M: Molecular weight marker (1kb); Lanes Lane M: Molecular weight marker (1kb); Lane +ve Positive control, -ve: negative control. Lane 1 until 12 are test samples for *CO1* gene fragments from DNA extracted from houseflies.

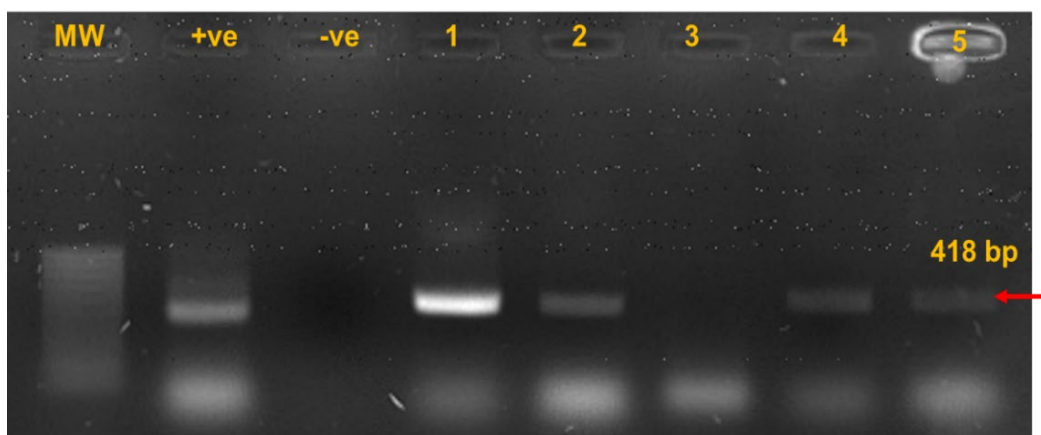


Figure 4.5 PCR amplification of *ITS2* gene. Lane M: Molecular weight marker (1kb); Lane +ve Positive control, -ve: negative control, Lane 1,2,3,4 and 5 are test samples for the amplification of the *ITS2* gene fragment.

4.3.3 Comparative analysis

To confirm if PCR fragments amplified with the different primers corresponded with *16S rRNA*, *CO1* and *ITS2* targeted gene sequences samples positive for each gene were sent to Inqaba Biotechnical Industries, Pty, Ltd in Pretoria for sequencing. The BLASTn homology search in GenBank showed that *Musca domestica 16S rRNA* sequences from the present study exhibited 99% nucleotide identity match with previously published sequences of *M. domestica* strains from other countries (Table 4.4). When one of the sequences from this study was aligned with a published sequence, four substitution mutations were identified with no gaps observed (Figure 4.4).

For *CO1*, the BLASTn homology search in GenBank showed that *M. domestica CO1* sequences determined in the current study exhibited 99% nucleotide identity match with previously published sequences of *M. domestica* strains (Table 4.5). When one of the sequences from this study was aligned with a published sequence, only three substitution mutations were identified, and no gaps were observed (Figure 4.5).

However, with *M. domestica ITS2* sequences determined in this study exhibited identities of 92% to 97% when compared to other published sequences (Table 4.6). The alignment of *M. domestica* sequence with a published sequence from Korea is shown in figure 4.6. The homology search showed that *M. domestica CO1* sequences

generated in this study exhibited 99% identity with published sequences of *M. domestica* sequences published in GenBank's NCBI database. The alignment of *M. domestica* ITS2 from the current study with published sequence from Thailand is shown in figure 4.6.

Table 4.3: BLASTn results of 16S rRNA gene from houseflies

Targeted gene	Blast description	Query cover	E-value	Identity	Accession number	Geographical distribution
16S rRNA	<i>M. domestica</i>	99%	0.0	99%	KT272823	Singapore
	<i>M. domestica</i>	99%	0.0	99%	KT272828	Singapore
	<i>M. domestica</i>	99%	0.0	99%	KT272829	Singapore
	<i>M. domestica</i>	99%	0.0	99%	KT272857	Singapore
	<i>M. domestica</i>	99%	0.0	99%	JQ246756	Brazil

Table 4.4 BLASTn results of CO1 gene from houseflies

Targeted gene	Blast description	Query cover	E-value	Identity	Accession number	Geographical distribution
CO1	<i>M. domestica</i> CO1	98%	0.0	99%	KR262647	Korea
	<i>M. domestica</i> CO1	92%	0.0	99%	MN609617	Korea
	<i>M. domestica</i> CO1	92%	0.0	99%	MN585203	China
	<i>M. domestica</i> CO1	92%	0.0	99%	HE614023	United Kingdom

Table 4.5 BLASTn results of ITS2 gene from houseflies

Targeted gene	Blast description	Query cover	E-value	Identity	Accession number	Geographical distribution
ITS2	<i>M. domestica</i> ITS2	91%	1e-132	93%	EF061808	China
	<i>M. domestica</i> ITS2	91%	3e-148	96%	EF061809	China
	<i>M. domestica</i> ITS2	91%	5e-142	93%	EF560189	Brazil
	<i>M. domestica</i> ITS2	91%	7e-155	95%	EU555394	China
	<i>M. domestica</i> ITS2	91%	5e-142	93%	EU555400	China
	<i>M. domestica</i> ITS2	91%	9e-164	97%	JQ811276	Thailand
	<i>M. domestica</i> ITS2	91%	1e-137	92%	JQ811278	Thailand
	<i>M. domestica</i> ITS2	91%	8e-140	93%	JQ811284	Thailand

Score	Expect	Identities	Gaps	Strand
996 bits(539)	0.0	549/554(99%)	0/554(0%)	Plus/Plus
Query 2		CCGGTCTGAACTCAGATCACGTAAGAATTTAAAAGTAGAACAGACTTAACATTTTAGCGG		61
Sbjct 12740		CCGGTTTGAACCTCAGATCATGTAAGAATTTAAAAGTCGAACAGACTTAACATTTTAGCGA		12799
Query 62		CTACACCAAAATATATCTCTTAATCCAACATCGAGGTCGCAATCTTTTTATCGATATGA		121
Sbjct 12800		CTACACCAAAATATATCTCTTAATCCAACATCGAGGTCGCAATCTTTTTATCGATATGA		12859
Query 122		ACTCTCCaaaaaaaTTACGCTGTTATCCCTAAAGtaacttaatttttaaatcgtaataa		181
Sbjct 12860		ACTCTCCAAAAAATTACGCTGTTATCCCTAAAGTAACTTAAATTTTAAATCGTTAATAA		12919
Query 182		cggatcaattattcataaattaatgatttaattaattaaaagttttttaaatTTAATAT		241
Sbjct 12920		CGGATCAATTATTATAAATTAATGATTTAATTAATTAAGTTTAAATTTTAAATTTAATAT		12979
Query 242		caccccaataaaatattaTCAACAATCACAAcaaaaaaaTCCACAAATTTGTAATTATC		301
Sbjct 12980		CACCCCAATAAAATATTATCAACAATCACAACAAAAAAATCCACAAATTTGTAATTATC		13039
Query 302		TAAATATAAGATTATAGGGTCTTCTCGTCTttttaatttttagctttttgactaa		361
Sbjct 13040		TAAATATAAGATTATAGGGTCTTCTCGTCTTTTAAATTTATTTTAGCTTTTGAATAA		13099
Query 362		aaaataaaattctattataaatttttatgaaaCAGTTAATATCTCGTCCAACCATTATA		421
Sbjct 13100		AAAAATAAATTCTATTATAAATTTTATGAAACAGTTAATATCTCGTCCAACCATTATA		13159
Query 422		CCAGCCTTCAATTAAGACTAATGATTATGCTACCTTTGCACAGTTAGGATACTGCGGC		481
Sbjct 13160		CCAGCCTTCAATTAAGACTAATGATTATGCTACCTTTGCACAGTTAGGATACTGCGGC		13219
Query 482		CATTTAAATAATTCAGTGGGCAGGTTAGACTTTTTAAATAATTCAAAAAGACATGTATT		541
Sbjct 13220		CATTTAAATAATTCAGTGGGCAGGTTAGACTTTTTAAATAATTCAAAAAGACATGTATT		13279
Query 542		TGTTAAACAGGCGA		555
Sbjct 13280		TGTTAAACAGGCGA		13293

Figure 4.6 BLASTn results showing the alignment of *M. domestica* 16S rRNA gene sequence from the current study. The subject sequence, *M. domestica* accession no KT272828 with 99% identity showing four mutations (two transition and two transversion mutation) identified by a red asterisk and no gap.

Score	Expect	Identities	Gaps	Strand
1107 bits(599)	0.0	605/608(99%)	0/608(0%)	Plus/Plus
Query 1	GATCTGGTATAGTAGGAACATCATTAAGAATTTTAATTTCGAGCTGAATTAGGACACCCTG	60		
Sbjct 26	GATCTGGTATAGTAGGAACATCATTAAGAATTTTAATTTCGAGCTGAATTAGGACACCCTG	85		
Query 61	GTGCTCTAATTGGAGACGATCAAATTTATAATGTTATTGTAACAGCTCATGCTTTTATTA	120		
Sbjct 86	GTGCTCTAATTGGAGACGATCAAATTTATAATGTTATTGTAACAGCTCATGCTTTTATTA	145		
Query 121	TAATTTTCTTTATAGTAATACCTATTATAAATTGGAGGGTTTGGAAATTGATTAGTTCCTT	180		
Sbjct 146	TAATTTTCTTTATAGTAATACCTATTATAAATTGGAGGGTTTGGAAATTGATTAGTTCCTT	205		
Query 181	TAATATTAGGAGCTCCAGATATAGCATTCCCTCGAATGAATAATATAAGTTTTTGATTAT	240		
Sbjct 206	TAATATTAGGAGCTCCAGATATAGCATTCCCTCGAATGAATAATATAAGTTTTTGATTAT	265		
Query 241	TACCTCCTGCATTAACTCTATTATTAGTAAGAAGTATAGTAGAAAAGGGAGCTGGAACAG	300		
Sbjct 266	TACCTCCTGCATTAACTCTATTATTAGTAAGAAGTATAGTAGAAAAGGGAGCTGGAACAG	325		
Query 301	GTTGAACTGTTTATCCACCTTTATCATCAATTATTGCTCATGGTGGAGCTTCAGTTGATT	360		
Sbjct 326	GTTGAACTGTTTATCCACCTTTATCATCAATTATTGCTCATGGTGGAGCTTCAGTTGATT	385		
Query 361	TAGCTATTTTCTCTCTTCACTTAGCAGGAATTTCTTCAATTTTAGGGGCAGTAAATTTTA	420		
Sbjct 386	TAGCTATTTTCTCTCTTCACTTAGCAGGAATTTCTTCAATTTTAGGAGCAGTAAATTTTA	445		
Query 421	TTACAACCTGTTATTAACATACGATCAACAGGAATTACATTTCGATCGAATGCCTTTATTG	480		
Sbjct 446	TTACAACCTGTTATTAACATACGATCAACAGGAATTACATTTCGATCGAATGCCTTTATTG	505		
Query 481	TTTGATCAGTTGTAATTACTGCattattattattattatCTCTTCCTGTTCTTGCTGGAG	540		
Sbjct 506	TTTGATCAGTTGTAATTACTGCATTATTATTATTATTATCTCTTCCTGTTCTTGCTGGAG	565		
Query 541	CTATCACTATACTATTAACATGATCGAAATTTAAATACTTCATTCTTTGATCCAGCTGGAG	600		
Sbjct 566	CTATTACTATACTATTAACATGATCGAAATTTAAATACTTCATTCTTTGACCCAGCTGGAG	625		
Query 601	GAGGTGAT 608			
Sbjct 626	GAGGTGAT 633			

Figure 4.7 BLASTn results showing the alignment of *M. domestica* CO1 gene sequence from the current study. The subject sequence, *M. domestica* accession no MN609617 with 99% identity showing three transition mutations identified by a red asterisk and no gap.

Score	Expect	Identities	Gaps	Strand
588 bits(318)	9e-164	338/347(97%)	3/347(0%)	Plus/Plus
Query 2	AAATAGGTTGCTT*	AAATAATCAAATCATCAAATGATTTGTGTTTTGAGCACATGTTATAT		60
Sbjct 41	AAATAAGTTGCTT	AAATAATCAAATCATCAAATGATTTGTGTTTTGAGCACATGTTATAT		100
Query 61	TACTGGATTCTGTT	CATCATTGATGATGAATAAAGAATTCATAATATTAATAGTCAATA		120
Sbjct 101	TACTGGATTCTGTT	CATCATTGATGATGAATAAAGAATTCATAATATTAATAGTCAAGA		160
Query 121	TAGAATACCTCATTGAAAAAATCCTAGTATTTTCTCAAATACTTATATA	tttttttCATT		180
Sbjct 161	TAGAATACCTCATTGAAAAAATCCTAGTATTTTCTCAAATACTTATATA	tttttttCATT		219
Query 181	CGATTGAGGAAGGTCTAGCATAAAATGA	tttttttATGAAACTATAATTGTCTCTTTTGA		240
Sbjct 220	CGATTGAGGAAGGTCTAGCATAAAATGATTTT	TTTATGAAACTAGAAATTGTCTCTTTTGA		279
Query 241	ATTGAAAAAC	AAAAATTCATATACCGAAAAGAAAGGAttttttCATTTGAAAATGAT		299
Sbjct 280	ATTGAAAAACA	AAAAATTCATATACCGAAAAGAAAGGATTTTTCATTTGAAAATGAT		339
Query 300	GGGGTTTCTATTTAATGTATAACTACTGAATAATTTCTGTTTTATAC			346
Sbjct 340	GGTGGTTTCTATTTAATGTATAACTACTGAATAGTATCTGTTTTATAC			386

Figure 4.8 BLASTn results showing the alignment of *M. domestica* ITS2 gene sequence from the current study. The subject sequence, *M. domestica* accession no JQ811276 covered 91% of the query sequence and it had 97% identity with three gaps and four mutations (two transition and two transversion mutation) shown by red asterisk.

4.3.4 Phylogenetic analysis of houseflies from South African hospices

The maximum likelihood tree topologies constructed with 16S *rRNA*, CO1 and ITS2 gene sequences of *Musca domestica* are shown in figures 4.7 to 4.9 respectively. Sequences from closely related species for 16S *rRNA*, CO1 and ITS2 genes retrieved from GenBank were also included in the phylogeny. Observations from tree topologies (16S *rRNA*, CO1 and ITS2) showed that *M. domestica* from the three sampled sites in the current study clustered together with other *M. domestica* from other countries with high bootstrap support values (>90%).

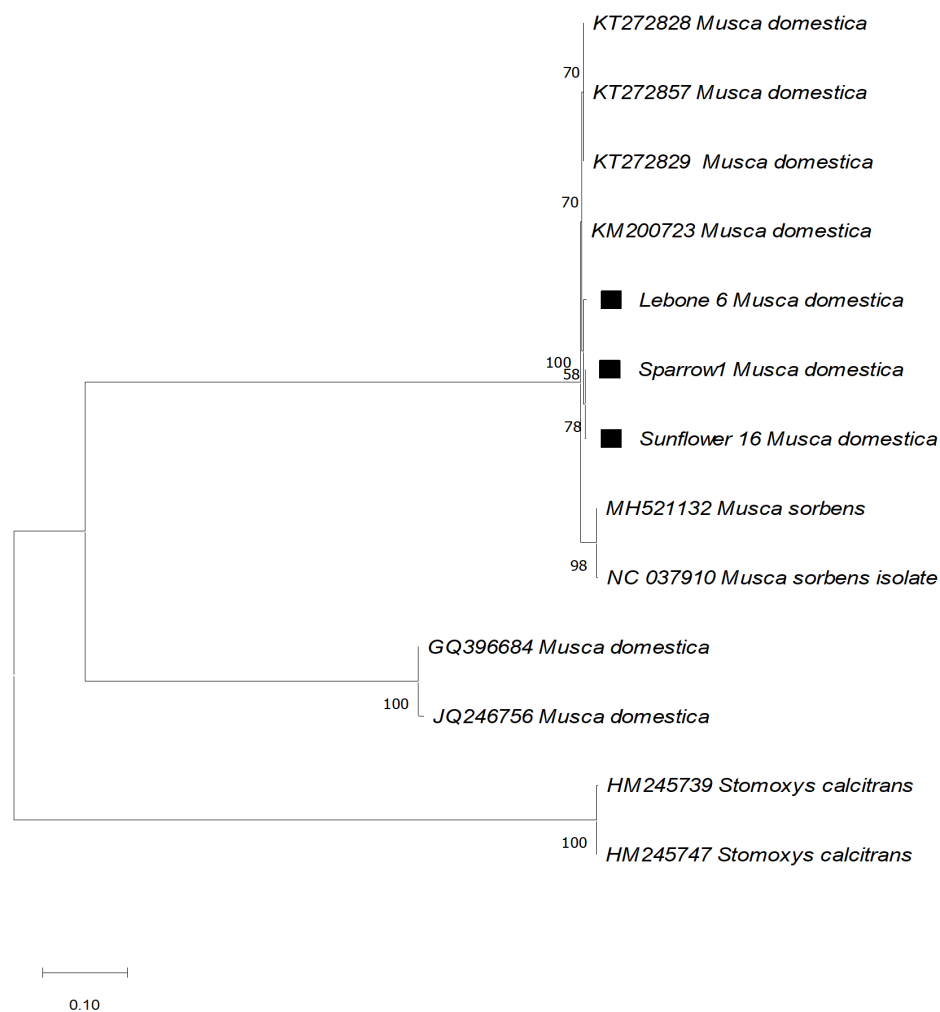


Figure 4.9 Molecular phylogenetic analysis by maximum likelihood (ML) method of the 16S *rRNA* gene. The tree highlights the position of houseflies from this study. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 13 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

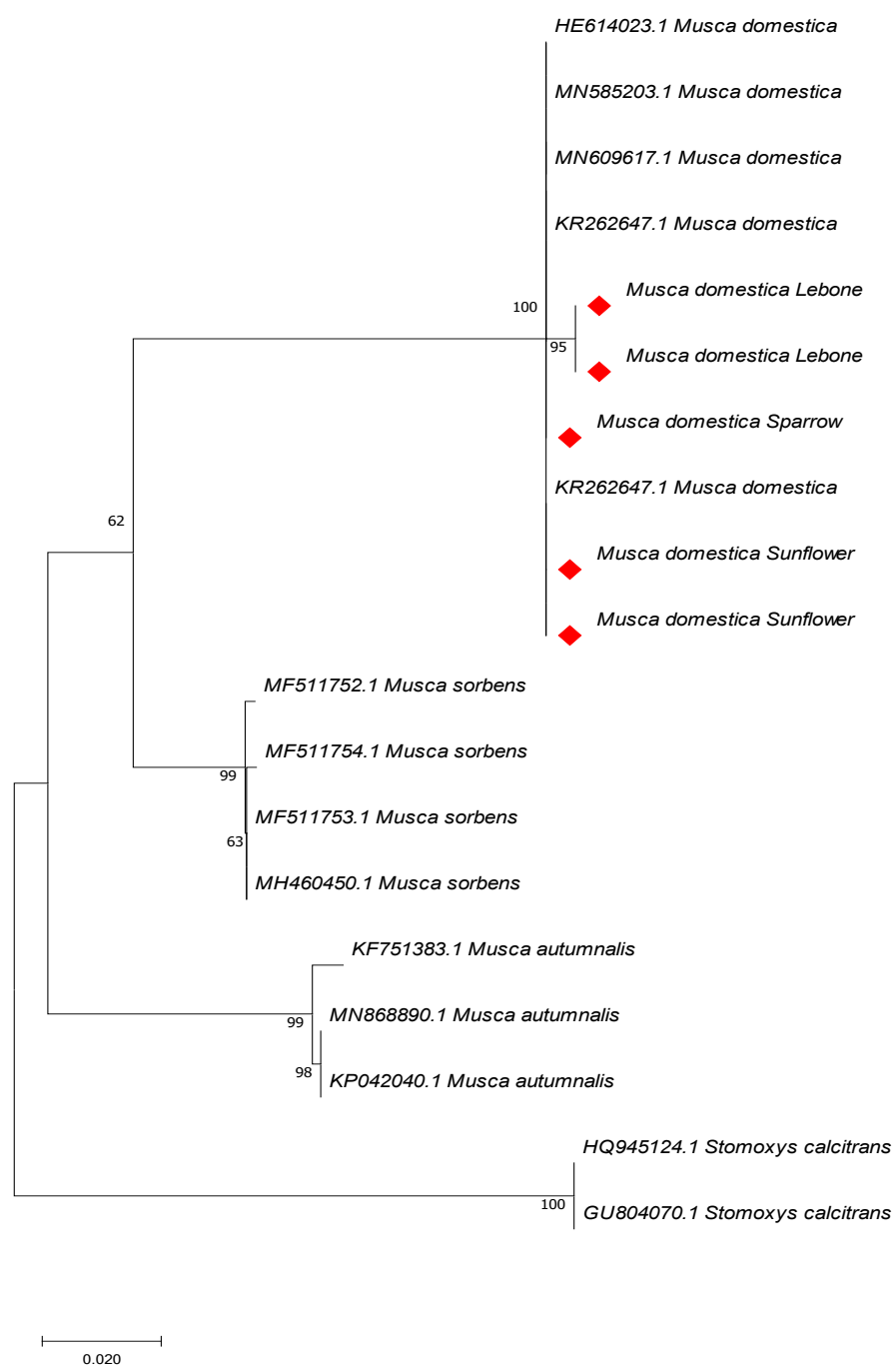


Figure 4.10 Molecular phylogenetic analysis by maximum likelihood (ML) method of the *CO1* gene. The tree highlights the position of houseflies from this study. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 19 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

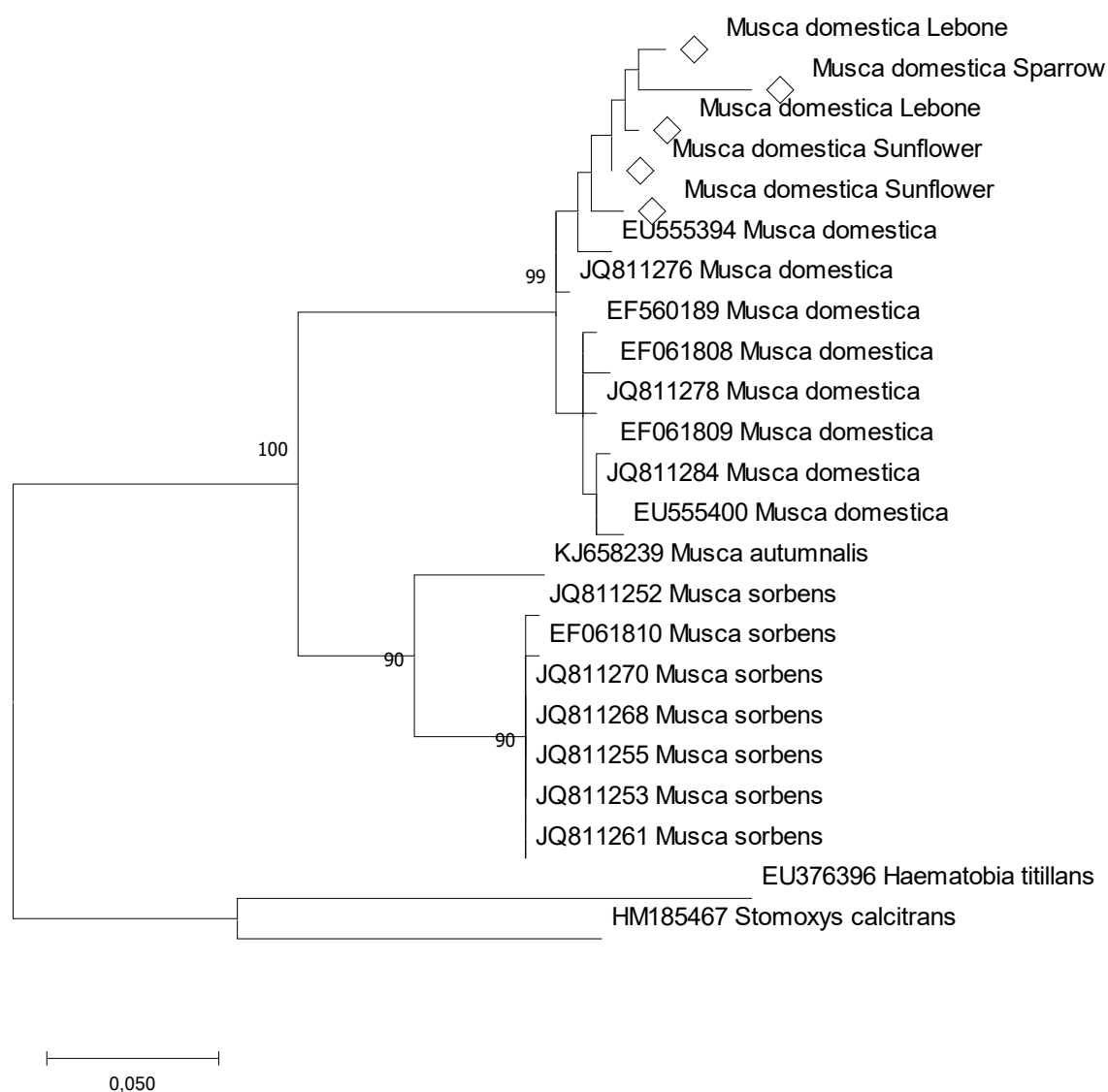


Figure 4.11. Molecular phylogenetic analysis by maximum likelihood (ML) method of the *ITS2* gene. The tree highlights the position of houseflies from this study. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018).

4.4 DISCUSSION

Musca domestica is a cosmopolitan insect pest of medical and veterinary importance in human and animal health, and it is morphologically and genetically diverse (Issa, 2019; Bosly, 2020). Thus, identification of *M. domestica* by medical entomologists and other scientists involved in the protection of public health is crucial (Bhakdeenuan *et al.*, 2012). In this study, *M. domestica* was identified using a stereo microscope with reference to documented literature based on the key features of adult housefly morphology and was further characterized genetically by the amplification of *16S rRNA*, *CO1* and *ITS2* gene regions to supplement morphological identification. Morphological identification keys for adult Muscidae include thorax, leg chaetotaxy and wing venation (Grzywacz *et al.* 2017; Bosly, 2020). According to our knowledge, this study reports for the first time on the identification of houseflies captured from hospices in South Africa.

Generally, the sample size of houseflies was higher in Lebone hospice as compared to other sampled hospice and this might mainly be due to the agricultural activities including animal husbandry that occur near the area. This type of agricultural activity, according to Nosal *et al.*, 2019, is associated with the accumulation of large amounts of manure, which plays a role in the development and dispersal of dipteran flies. Based on a personal observation, the Sunflower hospice is situated next to a hospital, the windows were always closed and hence the lower number of captured specimens. This can be considered as one of the control measures for houseflies. On the contrary, Sparrow Village, is situated next to a dumping site, giving exposure to garbage and other organic refuse which provides the main medium for housefly breeding site (WHO, 1997). This might justify the high numbers of muscoid collected in this area.

The molecular identification and phylogeny using species identification markers are regarded as efficient tools for the species identification (Ojha *et al.*, 2014). The results from the present study demonstrated the successful identification of *M. domestica* by both morphological and molecular amplification of *16S rRNA*, *CO1* and *ITS2* gene fragments, respectively. This confirmed the importance of considering both techniques (i.e., morphological and molecular) for identification of houseflies and other insects for taxonomic studies (Ajamma *et al.*, 2016). Sequenced PCR amplicons of the *16S*

rRNA, *CO1* and the *ITS2* genes fragments from this study matched with deposited *M. domestica* sequences available on GenBank database with 95% - 99% identity score which confirmed and supplemented observations made with the morphological identification. The application of DNA barcoding can delineate the limitations between species and allocate taxonomic status to unidentified species from known species. Thus, in the current study *16S rRNA*, *CO1* together with *ITS2* were used for DNA barcoding. According to Song *et al.*, (2008), the use of both *CO1* and *ITS2* genes is recommended as it provides a better resolution for identification among species. Hebert *et al.*, (2003) in their study on biological identifications through DNA barcodes have concluded that the rapidity of the evolution rates of mitochondrial genes were satisfactory to allow for discrimination between closely related species and phylogeographic groups (i.e., same species with different area of occupants) within the species. However, *CO1* has shown not to be satisfactory to differentiate among closely related calliphoridae species (Yusseff-Vanegas, 2017). Therefore, for accurate identification of species, GilArriortua *et al.*, (2014) have suggested the second nuclear internal transcribed spacer (*ITS2*) as a second barcoding locus for taxonomic species determinations in calliphorids. In this study, nuclear encoded gene *16S rRNA* was also included to support the accurate identification of the species. According to Guo *et al.*, (2012), this gene can infer relationships beneath the family level within the insects. Thus, in this study the monophyletic separation of *Musca* species was observed from the phylogenetic tree examined by *16S rRNA* confirmed the satisfactory resolution of the genetic marker. This is in accordance with the study by Guo *et al.*, (2012) where they observed the monophyletic separation of the five sarcophagid species from the phylogenetic tree.

According to the DNA barcode Consortium criteria for species identification, species in a phylogeny requires to share monophyletic association (Meyer and Paulay, 2005). From phylogenetic analysis targeting the *16S rRNA*, *CO1* and *ITS2* gene regions by means of maximum likelihood trees topology confirmed the identification of *M. domestica* specimens. Thus, a monophyletic group was observed showing close relationship among *M. domestica* strains generated from this study and various countries including China, Korea, Singapore and United Kingdom. This also showed a strong relation between houseflies, collected from the sampled hospices. These *M. domestica* clustered together with 99% support with *M. domestica* isolates obtained

from GenBank. High nodal support values on the constructed phylogenetic trees, demonstrate the suitability of *16S rRNA*, *CO1* and *ITS2* for species determination, which is the foundation of DNA barcoding.

In conclusion, the identification of houseflies using morphological and genetical analysis was shown to be successful in this study. This proved that the combination *16S rRNA*, *CO1* and *ITS2* genes are potential genetic markers that can be used in combination with morphology-based tools for accurate species identification of houseflies. Analysis made in this study showed that the housefly captured in this study form a well-supported clade with corresponding congener, however they showed the presence of genetic variation. Thus, more extensive analysis using more genetic markers are crucial to determine the population genetics of various *Musca* species in the South African context. Furthermore, they will aid in determination of the actual abundance and diversity of South African houseflies from various habitat niches.

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CHAPTER 5

DETECTION OF GUT MICROBIAL COMMUNITIES ASSOCIATED WITH HOSPICE HOUSEFLIES

5.1 INTRODUCTION

Association of bacterial communities and muscoid flies; especially, the housefly (*Musca domestica*) has been studied from numerous perspectives, including (i) the importance of microbes for larval development, (ii) digestibility of bacteria in the intestinal tract of fly larvae and (iii) the potential transmission of bacterial pathogens by adult flies (Zurek *et al.*, 2000). As such, these flies tend to be associated with microbe rich habitats including domestic and agricultural settings for reproductive purpose (Zurek and Nayduch, 2016). These settings are mainly packed with diverse and active microbial communities which create an ideal opportunity for houseflies to gather and potentially transmit pathogenic organisms to both human and animal food (Vazirianzadeh *et al.*, 2008; Gupta *et al.*, 2012). Macovei and Zurek, (2006), reported on the houseflies collected from food handling and serving facilities with the capacity to harbour as well as to transmit antibiotic resistant and virulent bacterial cells.

Adult houseflies are highly mobile, and they are capable of transporting bacterial cells from highly contaminated substrate to various substrates (Nazari *et al.*, 2017). Houseflies can transmit pathogenic microorganism through contamination of different parts of their external body surface, including feet, wings, appendages and mouth parts (Vazirianzadeh *et al.*, 2008; Gupta *et al.*, 2012; Zurek and Nayduch, 2016). Thus, a single housefly is cable to spread disease causing agents including pathogens causing infantile diarrhea, cholera, bacillary dysentery, tuberculosis, anthrax and ophthalmia (Vazirianzadeh *et al.*, 2008; Zurek *et al.*, 2000; Gupta *et al.*, 2012).

The close association of housefly and bacteria, and its role in pathogen transmission, makes it an ideal model organism to study the importance and variation of the microbiota of vector species (Bahrndorff *et al.*, 2017). To clearly understand the impact that the diverse microbial communities have on their hosts when these hosts serve as

a vector, is of importance to obtain better knowledge of these bacterial communities (Wei *et al.*, 2014).

Early studies of bacterial diversity were primarily based on cultivation methods. However, most bacterial pathogens are uncultivable especially outside their host environment (Wei *et al.*, 2014; Zhao *et al.*, 2017). Therefore, recent high-throughput DNA sequencing approaches provide means of characterizing bacterial communities and identifying both cultivable and uncultivable bacteria. This is done to provide an expanded perspective on bacterial diversity with higher coverage and a focus on a different set of organisms (Zhao *et al.*, 2017).

Due to close association among houseflies, animals and humans, these insects and their microbiome are of interest in this study. Housefly gut represents an interesting and unexplored niche for analyzing microbial ecology which will then offer opportunities for research concerning the impact of diverse and dynamic microbial communities on human health and disease (Gupta *et al.*, 2012). The study by Bahrndorff *et al.*, (2017) showed that analysis of gene sequences presented the most abundant classes of bacteria found across all farm sites including members of the phylum Bacilli, Clostridia, Actinobacteria, Flavobacteria, and all classes of Proteobacteria and at the genus level the most genera include *Corynebacterium*, *Lactobacillus*, *Staphylococcus*, *Vagococcus*, *Weissella*, *Lactococcus* and *Aerococcus*. Hence, this study aimed to explore the bacterial communities associated with the housefly captured from hospices in Bloemfontein and Johannesburg using the culture independent technique detecting 16S rRNA gene which is conserved amongst bacterial communities by Illumina MiSeq next generation sequencing platform.

5.2 MATERIALS AND METHODS

5.2.1 Study area, housefly sampling and DNA extraction

Study area, housefly sampling and identification as well as DNA extraction using Qiagen DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) have been described in detail under section 4.2.1, 4.2.2 and 4.2.3 of Chapter 4 respectively.

5.2.2 Partial 16S *rRNA* gene amplicon sequencing

Partial 16S *rRNA* gene (hypervariable V3-V4) libraries were amplified using the following universal bacterial primer pair 341F (5' -CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') (Klindworth *et al.*, 2013).

Briefly, the workflow for library preparing involved: an initial stage 'amplicon PCR' targeted at amplifying the area of concern by using 12.5 ng of genomic DNA, 2x KAPA HiFi HotStart ReadyMix [0.5 U DNA polymerase, 0.3 mM dNTPs, 2.5 mM MgCl₂] (Kapa Biosystems, Wilmington, MA, USA) and 5 µM of the above-mentioned primers. The PCR was performed at a final volume of 25 µl by denaturing at 95°C for 3 minutes, followed by 25 cycles of 95°C at 30 seconds, 55°C at 30 seconds and 72°C at 30 seconds. A final elongation step of 72°C was included for 5 minutes. Using Agencourt AMPure XP beads (Beckman Coulter Genomics, California, USA), amplicons were then subjected to a 'PCR clean-up' phase. After cleaning, index PCR attaching dual indexes (Nextera XT Index Kit) was performed using 5 µl of the initial PCR amplicon as template DNA, 5 µl of Illumina Nextera XT Index Primer 1 (N7xx), 5 µl of Nextera XT Index Primer 2 (S5xx), 25 µl of 2x KAPA HiFi HotStart Ready Mix, and 10 µl of PCR-grade water. The thermocycling conditions were 95°C for 3 minutes followed by 8 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds and a final elongation step of 72°C for 5 minutes. The index PCR products underwent a second step of 'PCR clean-up' with Agencourt AMPure XP beads as mentioned before. The partial 16S *rRNA* libraries were then quantified using a Qubit fluorometer (Qubit 3.0, Life Technologies, Malaysia), standardized, pooled to a final concentration of 20 pM and subsequently denatured in 0.2 N NaOH solution. The pooled libraries were further diluted to a final concentration of 6 pM, spiked with 10% PhiX control and heat denatured for 2 minutes before loading samples on the reagent cartridge of MiSeq V3 (Illumina, San Diego, CA, USA). After completing a paired end of 2 x 300 bp reads sequencing run on the Illumina MiSeq, demultiplexing and secondary reading analyses were performed using MiSeq reporter software (Illumina, San Diego, CA, USA).

5.2.3 Metagenomic data analysis

5.2.3.1 Bioinformatic and diversity analyses

Demultiplexed paired-end reads were checked for quality using FastQC software (version 0.11.5, Babraham Institute, United Kingdom). Reads were then denoised (i.e., further quality filtering, error correction and removal of chimeric sequences) and clustered into amplicon sequence variants (ASVs) by using the DADA2 denoiser (Callahan *et al.*, 2016) (via q2-dada2) integrated into the Quantitative Insight into Microbial Ecology version 2 (QIIME 2 2020.8) (Bolyen *et al.*, 2019)). The denoised reads were assigned taxonomy by the Silva 16S *rRNA* taxonomy (version 132) (Quast *et al.*, 2013) using a trained classifier of the V3-V4 region. Thereafter, singletons and non-bacterial taxa (e.g., archaea, mitochondria and chloroplast) were eliminated. Alpha diversity indices including the number of observed ASVs (ASV richness, Chao1 richness estimation, Shannon-Wiener index (H') and the species dominance (D) were computed in QIIME2 software.

Downstream statistical analyses were carried out using Microbiome Analyst (Dhariwal *et al.*, 2017; Chong *et al.*, 2020) online web tool with Marker Data Profiling (MDP) module. A total of 1 720 766 ASVs were recovered after removing the singletons. Further filtering with default parameters yielded 273 ASVs. The normalization was carried out to rarefy the data to the minimum library size (14 282) using the total sum scaling option. Alpha diversity was calculated using Observed, Chao1 and Shannon as well as Simpson diversity measures. The beta-diversity comparisons were visualized by PCoA analysis ($\geq 97\%$ similarity) using Bray Curtis Index ($P < 0.05$; PERMANOVA). Metacoder software (Foster *et al.*, 2017) was implemented in R (R Core Team 2020) (ver., 3.6.1) for the construction and visualization of heat tree using the ASVs and taxonomy file from QIIME 2.

5.2.3.2 Data availability

The datasets generated during and/or analyzed during the current study were deposited in the NCBI Sequence Read Archive (SRA): SRP356827 under the BioProject: PRJNA800672.

5.3 RESULTS

5.3.1 Summary of generated ASVs

Four thousand eight hundred and seventy-one (4871) ASVs were generated from the Illumina MiSeq sequencing platform. A total of 1 720 766 reads were obtained with an average count of 39 108 per sample. Using the Microbiome Analyst for data analysis following the filtration step, 589 low abundance features and 14 low variance features were removed based on the interquartile range. The remaining features after the data filtering step were 121. In this study, FASTQ sequences obtained from housefly samples obtained in hospices were classified into phyla, classes, orders, families, and genera.

5.3.2 Taxonomic classification of bacteria in housefly

The microbiota composition at the phylum level detected from the three sampled hospices in both cities, Bloemfontein and Johannesburg was at the total of three phyla. The most abundant phyla detected with variable abundance observed among all houseflies were Proteobacteria, followed by Firmicutes and Bacteroidetes (Figure 5.1). The microbiota composition associated with the houseflies at the class level were assessed and there was a total of four bacterial classes observed in this study (Figure 5.2). The observation revealed that the most abundant class detected from all the three hospices in both cities were Gammaproteobacteria, Bacilli, Bacteroidia and Alphaproteobacteria. The class Alphaproteobacteria was observed in some of the samples from Lebone and Sparrow hospices and one sample from Sunflower.

Ten bacterial orders were identified in houseflies obtained in three hospices from Bloemfontein and Johannesburg. The dominant orders detected included Enterobacteriales, Lactobacillales, Cardiobacteriales, Flavobacteriales and Rhizobiales (Figure 5.3). The remaining orders all had few representatives showing a very low relative abundance. These included the following bacterial orders; Pseudomonadales, Caulobacterales, Orbales and Coxiellales.

Enterobacteriaceae was the most dominant bacterial family observed in all housefly samples obtained in hospices from Bloemfontein and Johannesburg. This dominant family was followed by other families including, Enterococcaceae, Dysgonomonadaceae, Weeksellaceae, Cardiobacteriaceae, Streptococcaceae, Rhizobiaceae and Leuconstocaceae to list the few (Figure 5.4).

The microbiota composition associated with the houseflies' content at the genus level were assessed from both cities, namely, Bloemfontein and Johannesburg. *Providencia* was the most abundant bacterial genera from all analyzed houseflies' samples, followed by *Enterobacter*, *Dysgonomonas*, *Escherichia-Shigella*, *Klebsiella* and *Morganella* (Figure 5.5). Additionally, *Coxiella*, *Enterococcus*, *Ignatzschineria*, *Subtonella*, *Wohlfahrtimonas*, *Serratia*, *Streptococcus*, *Proteus* and *Pseudomonas* and were also detected in most housefly samples

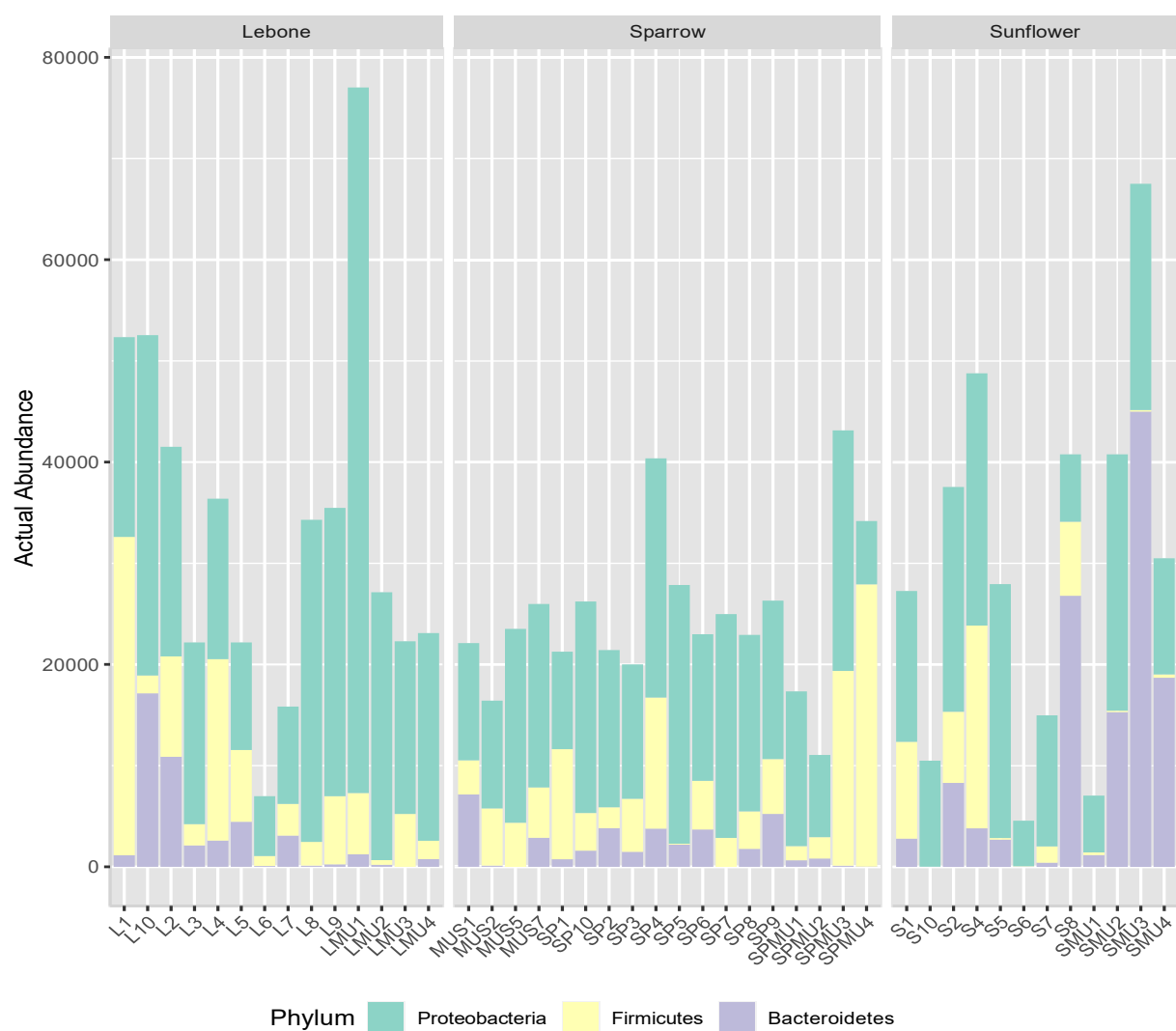


Figure 5.1 A stacked bar plot showing relative abundance of bacterial phyla detected from houseflies collected from three hospices in Bloemfontein and Johannesburg cities.

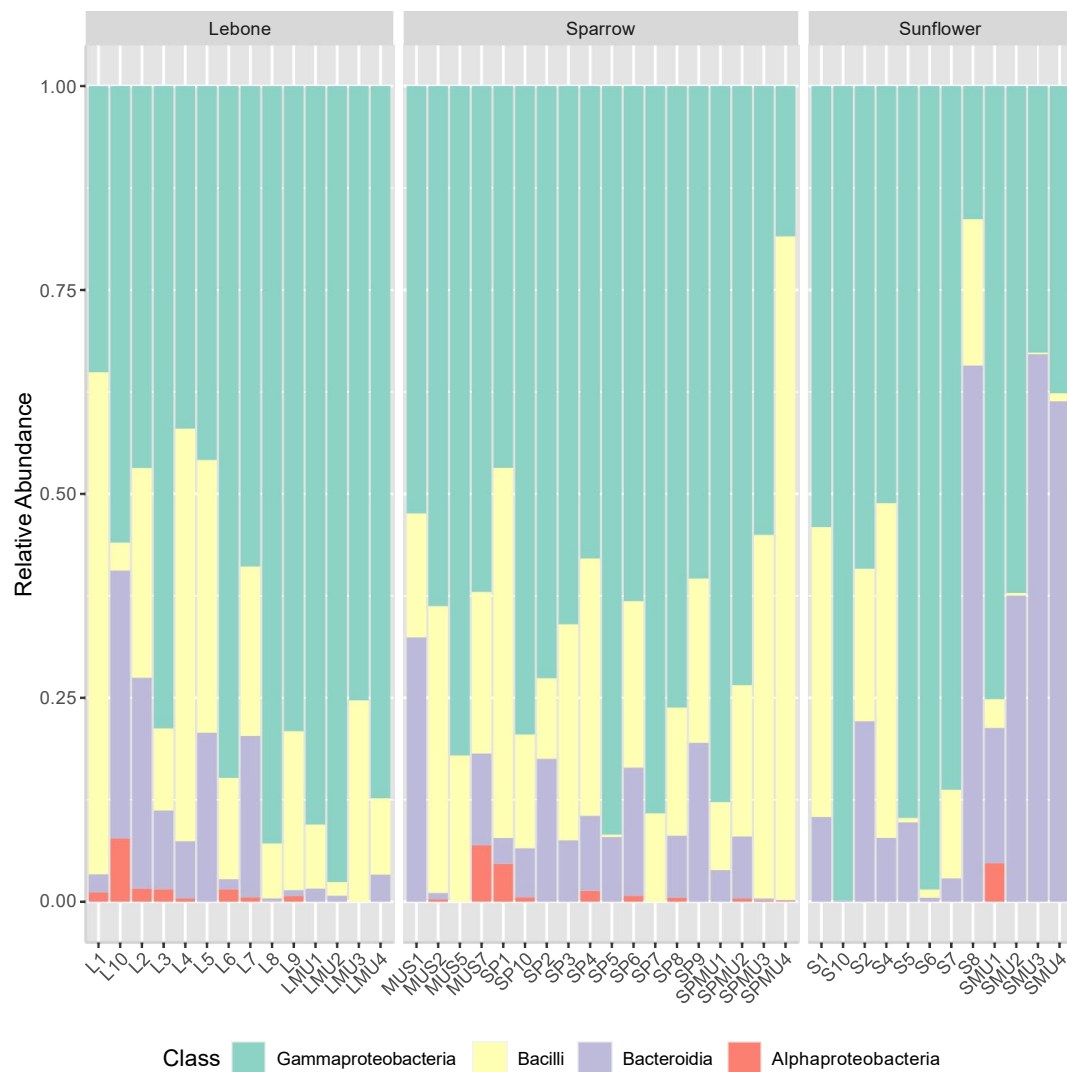


Figure 5.2 A stacked bar plot showing relative abundance of bacterial class detected from houseflies collected from three hospices in Bloemfontein and Johannesburg cities.

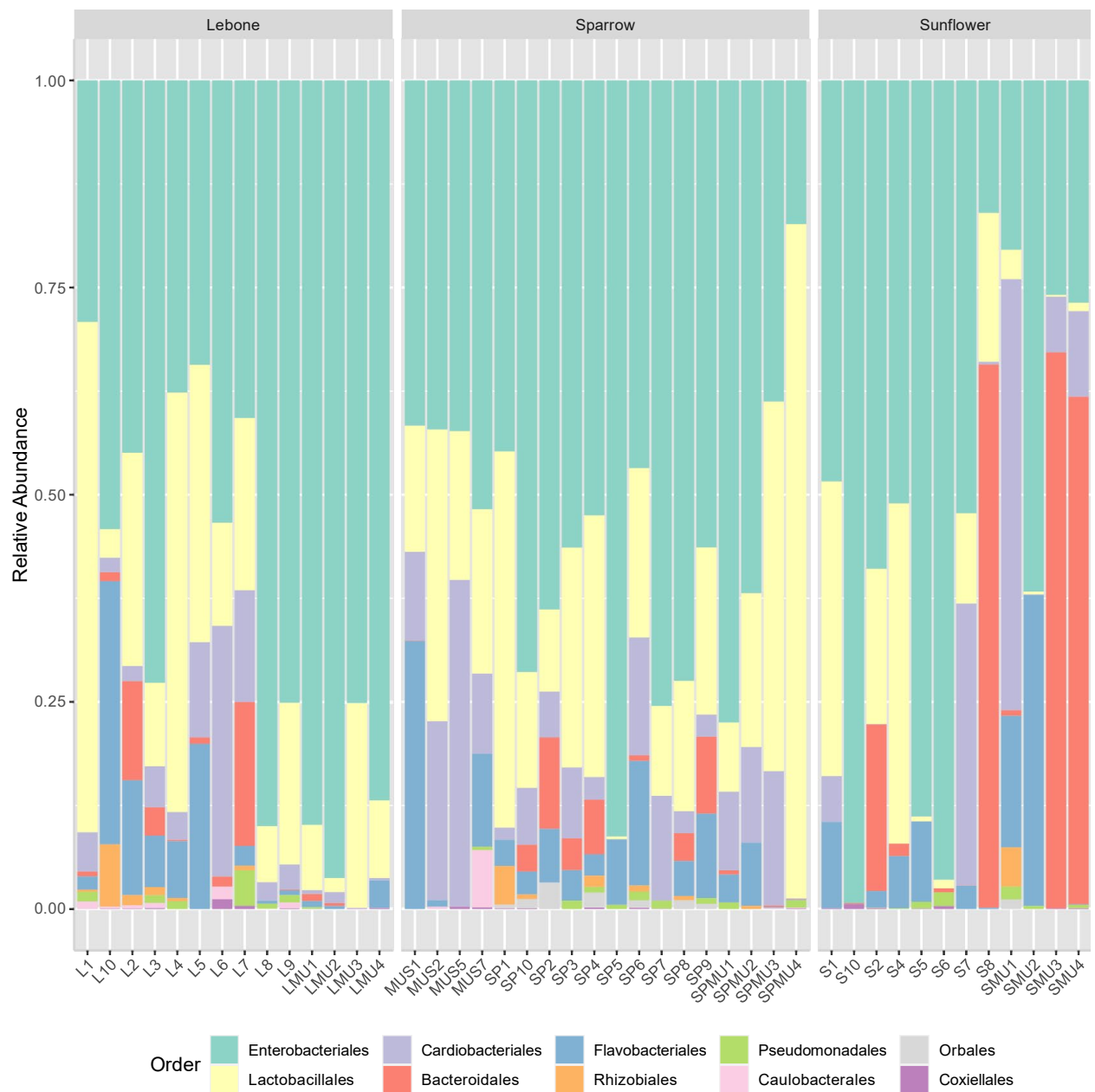


Figure 5.3 A stacked bar plot showing relative abundance of bacterial order detected from houseflies collected from three hospices in Bloemfontein and Johannesburg cities

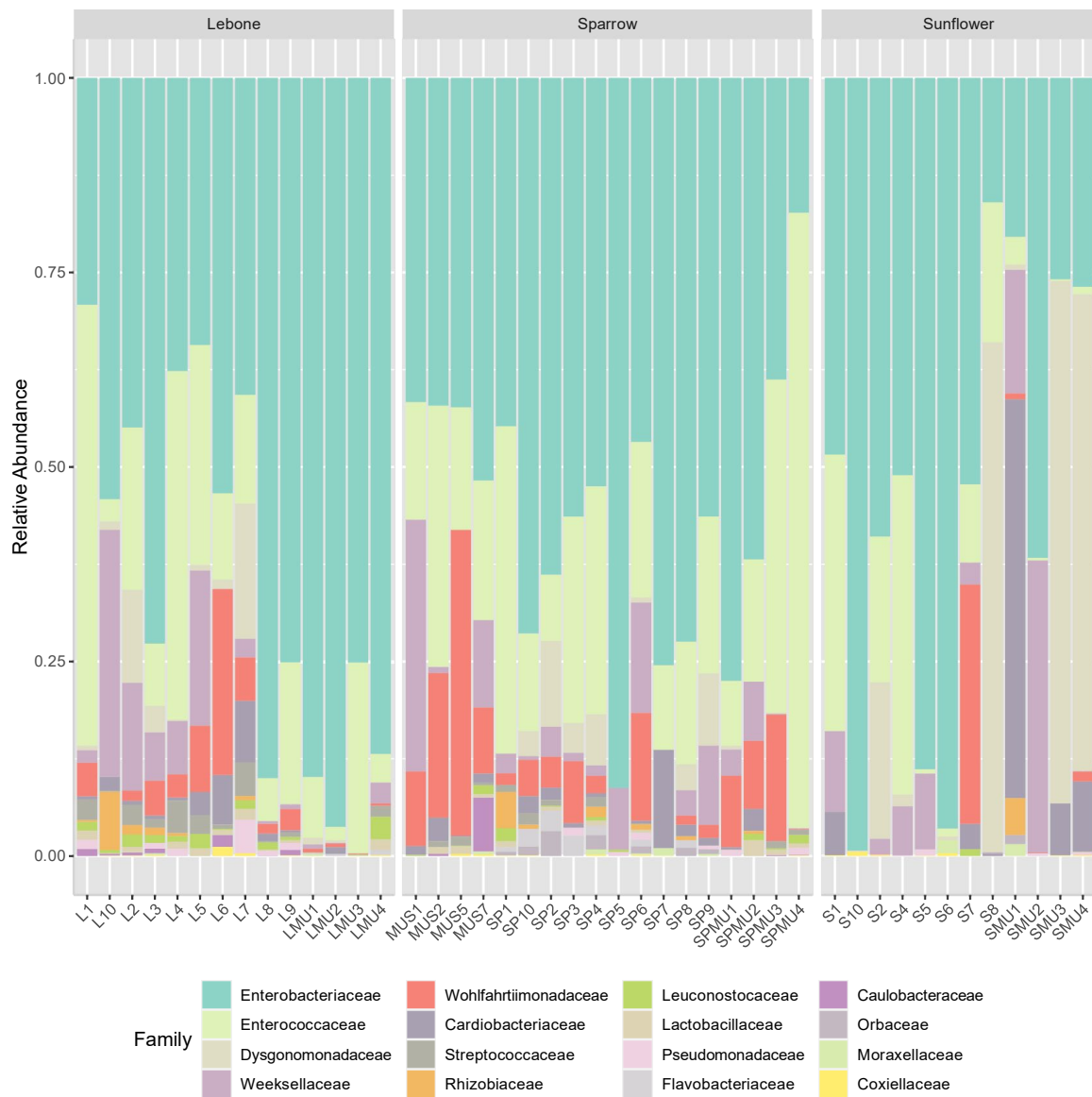


Figure 5.4 A stacked bar plot showing relative abundance of bacterial families detected from houseflies collected from three hospices in Bloemfontein and Johannesburg cities.

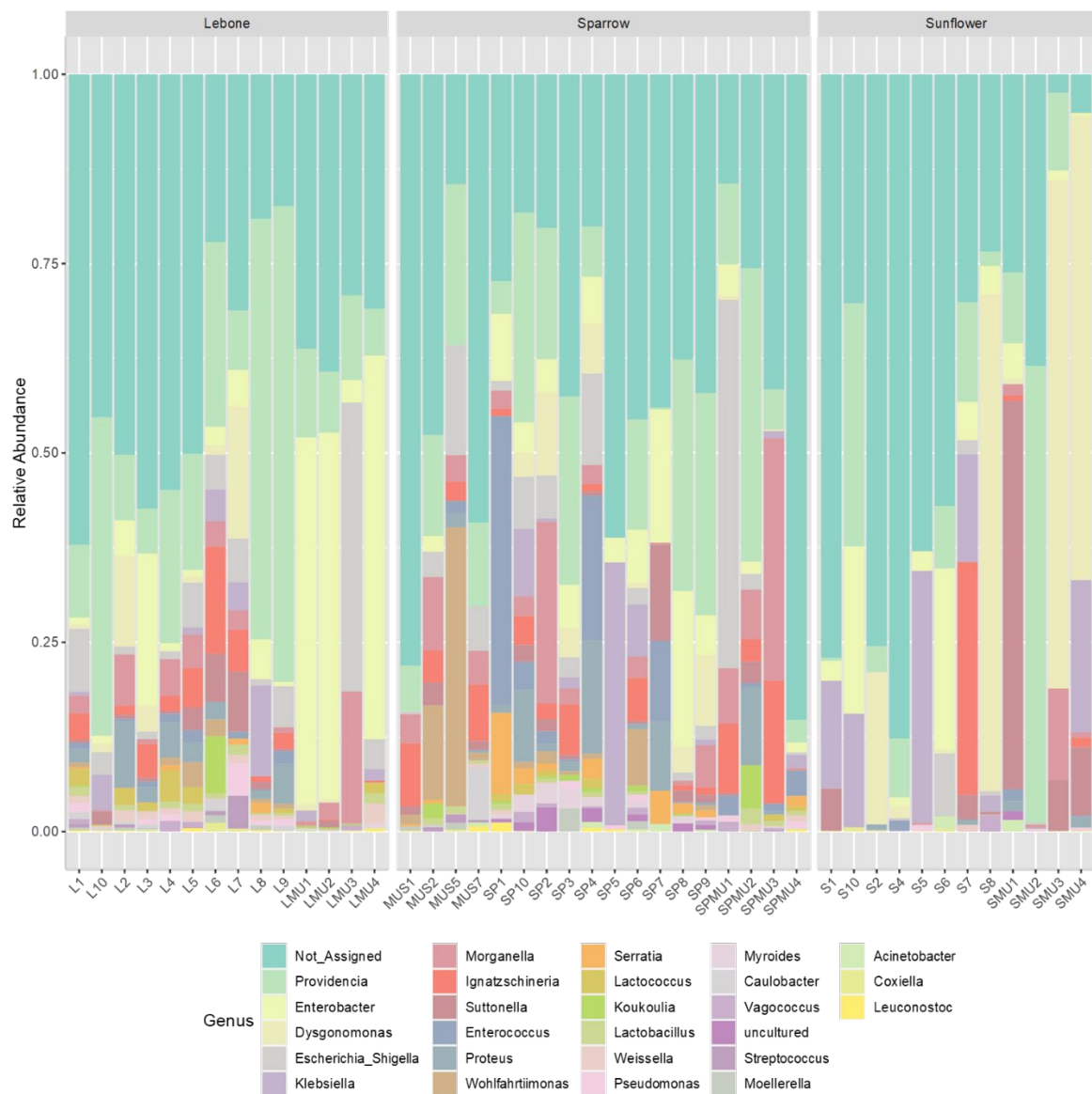


Figure 5.5 A stacked bar plot showing relative abundance of bacterial genera detected from houseflies collected from three hospices in Bloemfontein and Johannesburg cities.

5.3.3 Alpha diversity index

To assess the effect of sampling environment on alpha diversities, housefly samples from three hospices in different cities were compared. The observations from Chao1 indices showed the variation in species abundance and richness of housefly bacterial communities from individual samples (Figure 5.6). The samples from Lebone and Sparrow hospice were significantly more diverse than the samples from Sunflower hospice which appeared to have some low microbial communities as compared to other hospice samples.

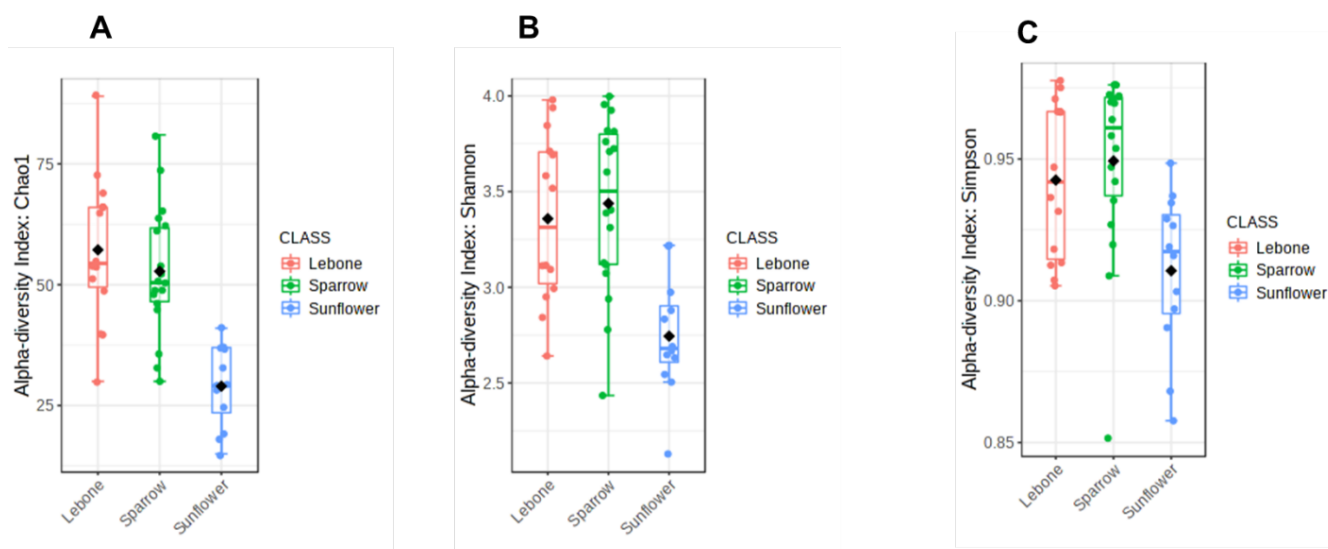


Figure 5.6 Boxplots showing the alpha diversity comparisons of housefly samples from hospices. A: Chao1 index; B: Shannon index; C: Simpson index.

5.3.4 Beta diversity index

Community ASV comparisons were visualised by Principal Coordinates analysis (PCoA) using Bray Curtis Index (Figure 5.7 and 5.8). Bray Curtis index which is used to quantify the compositional dissimilarity between two different sites, based on counts at each site showed no obvious clustering apparent within or between housefly samples obtained in both cities including Bloemfontein and Johannesburg. This observation reveal that the housefly samples share some of the bacterial species if not all. The differences in multivariate space among hospices in Bloemfontein and Johannesburg are significant (PERMANOVA $p < 0.05$; Figure 5.7). A dendrogram generated (Figure 5.8) showed that ASVs clustered together by location.

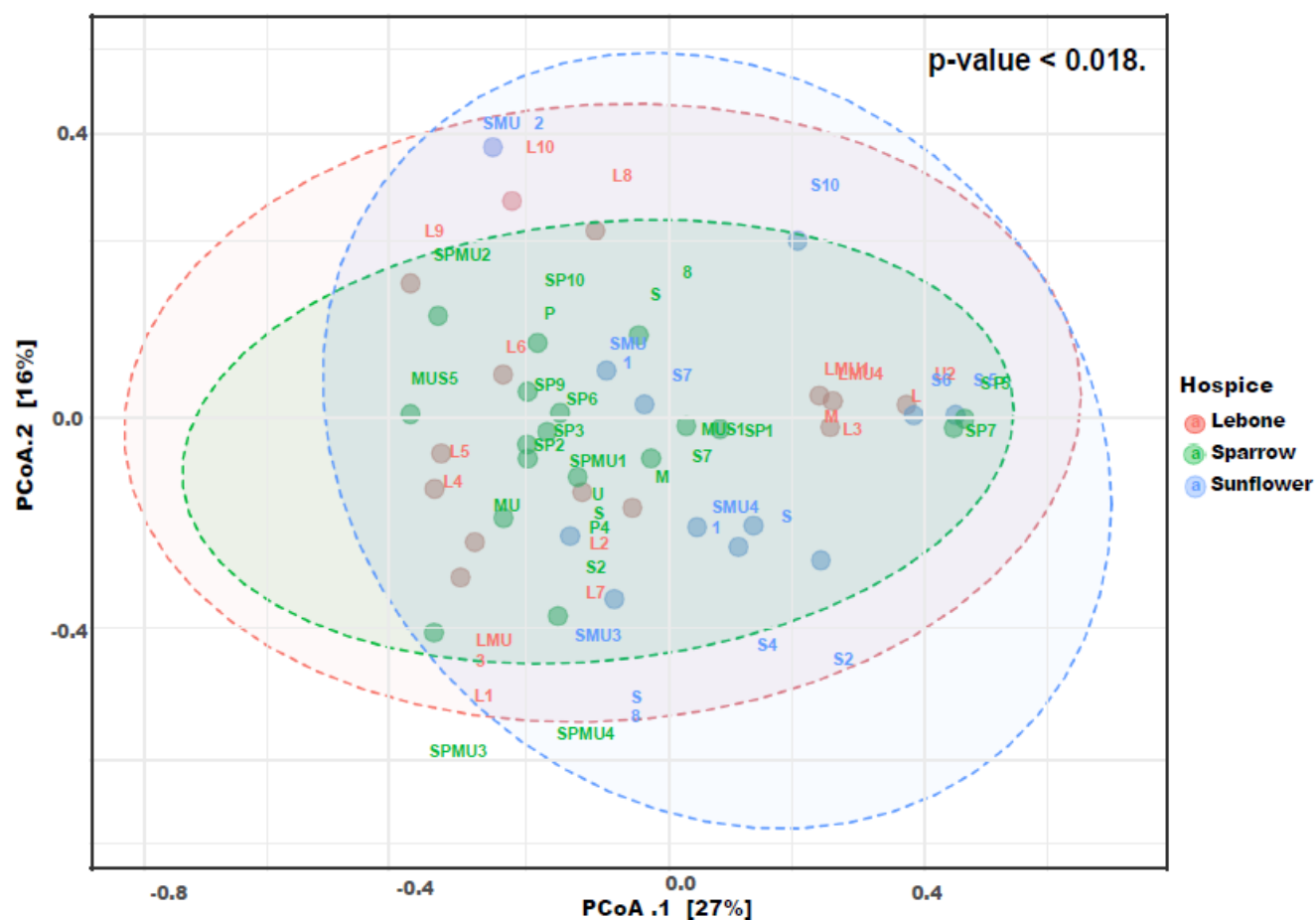


Figure 5.7 Multivariate differentiation of bacterial communities in hospices statuses. Dotted lines show the distance of every sample to its group centroids in multivariate space, while ellipses show 95% confidence intervals (standard error) in multivariate space around group centroids. The PERMANOVA P -values are also indicated in this figure.

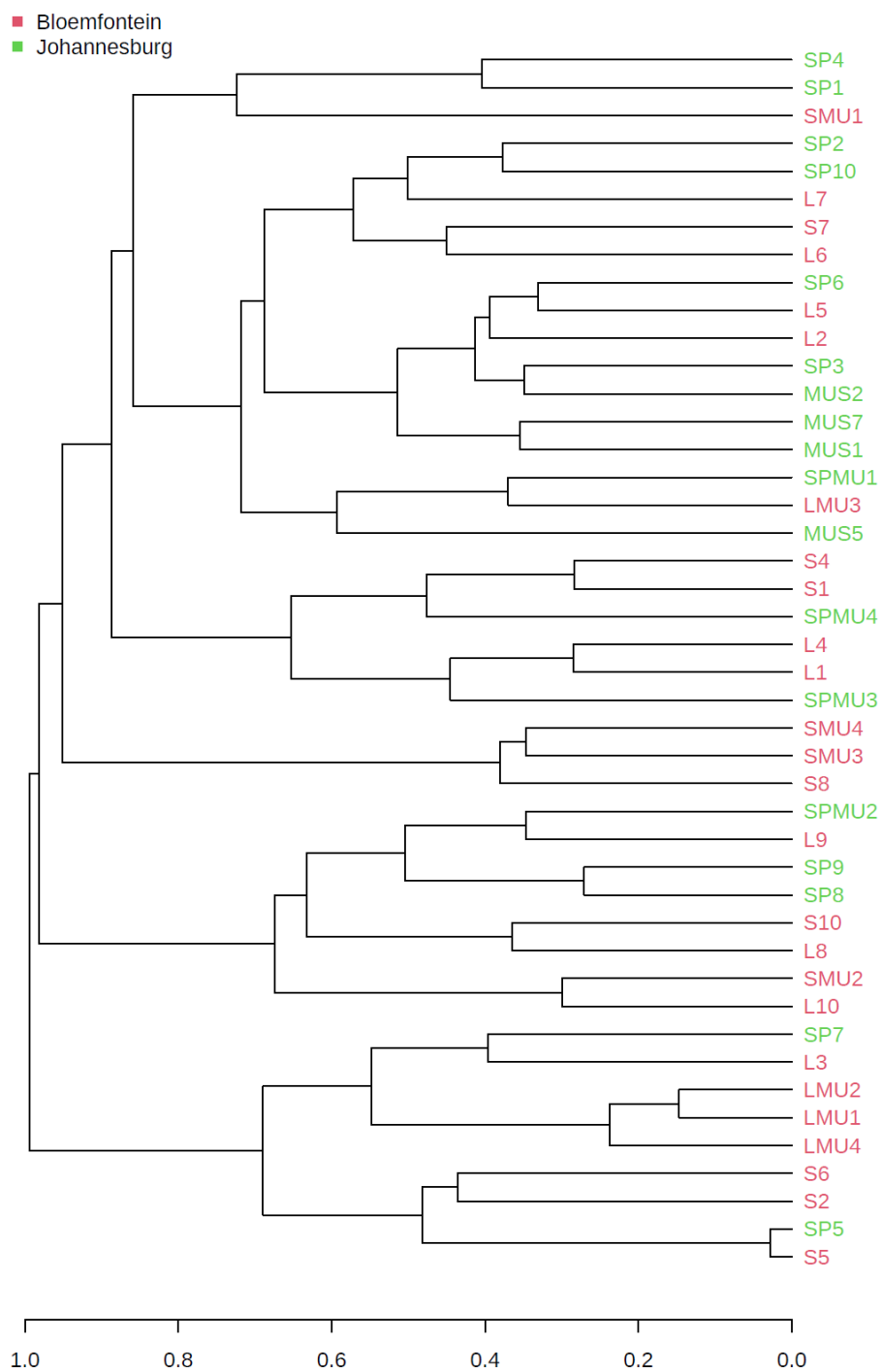


Figure 5.8 Cluster dendrogram showing how the ASVs from various locations clusters. Clustering was by location. Bray Curtis index measure and ward clustering method was used to generate the dendrogram.

5.4 DISCUSSION

It is important to investigate the complete bacterial population, including strains that cannot be cultivated under laboratory conditions and assess the persistence of antibiotic-resistant bacteria in a microbiota (Wei *et al.*, 2013). A considerable amount of data has been published regarding the diversity of bacteria in flies with a culture-based method (Wei *et al.*, 2013). However, this study explored the bacterial communities associated with houseflies from hospices in two of major cities of South Africa. The results provided a culture-independent description of the microbiota of a vector (*Musca domestica*) of public health importance. This work was designed to determine which bacterial communities are associated with houseflies obtained in hospices that may be associated with where food is prepared and consumed. The results obtained in this study have shown that houseflies captured from the different hospices are associated with highly diverse microbial communities. These findings correspond to observations made by Park *et al.*, (2019) where they assessed the potential influence of geography and habitat on the internal and external microbial communities of houseflies. The high species richness of the housefly microbial communities is likely to reflect the nature of houseflies, including feeding, breeding and living by animal manure (Bahrndorff *et al.*, 2017).

The abundant phyla detected in this study included Proteobacteria, Firmicutes, and Bacteroidetes. Similar results were obtained by Bahrndorff *et al.*, 2017 where they assessed houseflies sampled within and between farms. Also, a study by Zhao *et al.*, (2017) indicated that these bacterial phyla were present and dominant in the larval stage of houseflies. However, de Jonge *et al.*, 2020 have demonstrated the changes in the microbial community composition through the different housefly developmental stages, which was characterized by a diversification of colonisation during the larval stage of development, followed by an exchange of new microbiota. The change observed was of highly abundant colonisation by Bacteroidetes on adult flies to Firmicutes during the developmental stages and a combination of Bacteroidetes, Firmicutes and Proteobacteria from adult housefly samples. Microbial phyla observed in this study have been found to be associated with several other species of arthropods including *Manduca sexta* (Tobacco hornworm), *Helicoverpa armigera* (Cotton

bollworm), *Aedes albopictus* (Asian tiger mosquito), *Apis mellifera* (Western honey bee), *Culex quinquefasciatus* (southern house mosquito) and flesh fly (*Sarcophaga* sp.) and blowflies (Calliphoridae) (Brinkmann *et al.*, 2008; Priya *et al.*, 2012; Gupta *et al.*, 2012; Chandel *et al.*, 2013; Hendriksma *et al.*, 2013; Moro *et al.*, 2013; Gupta *et al.*, 2014; Junqueira *et al.*, 2017).

The class Gammaproteobacteria, Bacilli, Alphaproteobacteria and Bacteroidia were observed from the study by Bahrndorff *et al.*, (2017) and were the most dominant in this study. However, members of the family Enterobacteriaceae were the most dominant bacteria detected from the sampled houseflies in this study followed by Enterococcaceae, Dysgonomonadaceae, Weeksellaceae, Cardiobacteriaceae, streptococcaceae and Rhizobiaceae. Thus, the findings in this study are consistent with previous findings observed in the gut of wild caught houseflies (Gupta *et al.*, 2012). The same Enterobacteriaceae family was also found to be dominant in the gut of the flesh fly (Gupta *et al.*, 2014) and gut and reproductive organs of some fruit fly species (Wang *et al.*, 2014).

In this study, multiple species of bacteria were identified to genus level which are present in some housefly samples from some hospices. The prevalent genera identified in this study included *Providencia*, *Enterobacter*, *Dysgonomonas*, *Escherichia-Shigella*, *Klebsiella*, *Pseudomonas* and *Streptococcus* which are known to harbour potentially pathogenic species of clinical and veterinary relevance. These findings are in accordance with the systematic review by Khamesipour *et al.*, (2018) who made an observation of dominant pathogenic bacteria identified in houseflies captured from different environments. The study by Park *et al.*, 2019, observed genera *Streptococcus* and *Escherichia-Shigella* to be more prevalent in hospital fly samples and farm fly samples respectively.

The *Providencia* is a genus of ubiquitous Gram-negative bacteria in the family Enterobacteriaceae and cause several human diseases (Gupta *et al.* 2012; Zhao *et al.*, 2017). Members of the genus *Providencia* have been isolated from a range of

environmental niches and living organisms. *Providencia* is comprised of other opportunistic pathogens of humans and insects (Park *et al.*, 2019). However, this genus has been identified as part of the normal human gut flora (Zhao *et al.*, 2017). However, *Providencia* has been reported to be beneficial in carrion feeding insects including blow flies (Singh *et al.*, 2014). These genera were found to have nutritional benefits by producing several xylanases that help in decomposition of xylan observed at decomposition sites (Singh *et al.*, 2014).

Firmicutes were also a major component in the gut of housefly obtained in this study. The genus *Staphylococcus* from the Firmicutes phylum were not detected from the housefly samples in this study, however the observation of this genus was made by the other studies on houseflies in United states (Grübel *et al.*, 1997), India (Gupta *et al.*, 2012) and again United states (Zurek *et al.*, 2000). Genera *Streptococcus* and *Micrococcus* were more prevalent in hospital fly samples whereas *Clostridium* and *Escherichia-Shigella* were more prevalent in farm fly samples (Park *et al.*, 2019). However, there were no *Clostridium* and *Micrococcus* genera detected in this study.

The present study showed the diverse composition of bacterial communities in the gut of houseflies obtained in a hospice within the two large cities including Bloemfontein and Johannesburg. However, the study by Park *et al.*, 2019, has shown that microbial diversity of lab-grown flies was low as compared to wild flies obtained. Thus, the low microbial colonization of lab flies could indicate that housefly may survive with very low amounts of bacteria in their gut (Park *et al.*, 2019). It is noted that microbiota present in arthropods plays an important role in host nutrition and may possibly influence the transmission of vector borne pathogens (Bahrndorff *et al.*, 2017). Additionally, the importance of the environment in establishing the fly microbiota should be of interest in shaping microbiota of the housefly and linking microbial communities within the environment (Bahrndorff *et al.*, 2017; Park *et al.*, 2019). Since the housefly lives in close association with its microbiota (Bahrndorff *et al.*, 2017).

The presence of potential human pathogens identified in houseflies from hospices is of great concern since the insects may play a role in the dissemination of antibiotic resistant bacteria. Thus, these pathogens can cause serious and life-threatening

diseases in human and animals. The present study has examined the microbiota of adult *M. domestica* from the hospices and showed the diverse composition of bacterial communities in the gut of houseflies, with some genera being pathogenic to humans. Thus, the presence of houseflies in environment including hospices present a health hazard of pathogenic bacteria to the patient housed within the institution and the surrounding community. Therefore, it is of importance to limit fly breeding by employing proper sanitation to reduce the chances of bacterial infections among the community.

5.5 REFERENCES

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CHAPTER 6

MOLECULAR DETECTION OF BACTERIAL ANTIBIOTIC RESISTANCE AND VIRULENCE DETERMINANTS FROM HOUSEFLIES' DNA

6.1 INTRODUCTION

Musca domestica from the family Muscidae is an excellent vector of pathogenic bacteria including those that harbours antibiotic-resistant strains to residential settings (Macovei and Zurek, 2006). This is due to (i) their ability to develop in decaying organic material, (ii) their dependence on a live microbial community, (iii) their feeding mechanism (regurgitation), (iv) attraction to human food, as well as (v) their ability to fly long distances (Macovei and Zurek, 2006).

The transmission of antibiotic-resistant pathogens becomes easier because medically relevant bacteria are carried on the surface of the housefly's exoskeleton including legs, or mouthparts as well as in the alimentary canal (Onwugamba *et al.*, 2018). The mechanism of bacterial transmission by houseflies involves feeding on faecal material contaminated with pathogens. Subsequently, the regurgitation of pathogen contaminated digestive fluids or faecal excreta on human food which is eventually ingested by oblivious people (Osato *et al.*, 1998; Graczyk *et al.*, 2001). The risk of houseflies in distributing resistant bacterial strains from livestock and poultry farms to the public is of increasing concern (Zhang *et al.*, 2018). Hence, antibiotic resistance genes and virulence genes are regarded as emerging environmental contaminant (Pruden *et al.*, 2006).

Certain bacterial strains contain virulence factors that allow them to cause disease and evade host immune defenses. Thus, virulence in this context is referred to as the capability of a microorganism to cause damage in a host (Baceiro *et al.*, 2013; Diard and Hardt, 2017). These virulence factors are encoded by pathogenicity islands

(distinct genetic elements on the chromosomes) which largely depends on the environmental setting in which the bacterium lives (Gal-Mor and Finlay, 2006).

Many bacteria produce siderophores including enterobactin produced by enteric bacteria for iron (Fe) acquisition. The efficacy of this iron-scavenging system has resulted in the evolution of host resistance mechanisms including lipocalin-2. This Lipocalin-2, the host protein can bind and sequester enterobactin, thereby inactivating the molecule and impairing bacterial Fe acquisition (Begg, 2019). However, bacterial strain including non-typhoidal *Salmonella* serovar, *Salmonella* Typhimurium and *Bacillus anthracis* have evolved molecular derivatives of enterobactin, salmochelin and petrobactin respectively, which cannot be bound by lipocalin-2, therefore allowing continued Fe acquisition and increased virulence in murine infection models (Crouch *et al.*, 2008; Begg, 2019).

However, the severe Fe limitation experienced by bacterial pathogens during infection is also a key environmental signal. Thus, a ubiquitous environmental organism and or an opportunistic pathogen including *Pseudomonas aeruginosa*, utilises the restriction of Fe bioavailability as one mechanism to distinguish between environmental niches (Begg, 2019). Also, polyamines in bacteria can scavenge iron and free radicals, leading to acid resistance, promote biofilm formation, regulate expression of virulence factors, and mediate immune evasion during the establishment of bacterial infection (Banerji *et al.*, 2021).

Antibiotic resistance genes have been discovered to occur as part of multiple antibiotic-resistant super integrons. Thus, bacterial cells can contain antibiotic-resistant gene cassettes with one or more antibiotic resistance genes (Aarts *et al.*, 2001; Pruden *et al.*, 2006). Integrons are among the major types of mobile elements known to be involved in the capturing, mobilization, dissemination and expression of antibiotic resistance genes in bacterial communities (Makowska *et al.*, 2016; Ali *et al.*, 2020). Thus, these genetic elements play a major role in bacterial evolution, including the development of antimicrobial resistance (Ali *et al.*, 2020).

Molecular techniques including polymerase chain reaction (PCR) and quantitative real-time PCR (qPCR) with the aid of species-specific oligonucleotide primers and probes, have been developed for the detection of antibiotic resistance genes in bacteria (Czekalski *et al.*, 2014). The use of PCR to detect the presence of antibiotic resistance genes in a bacterial isolate or samples from different environments has been made familiar (Anjum *et al.*, 2017). Schwartz *et al.*, (2003), used the PCR technique to monitor for the presence of *vanA* encoding vancomycin resistance, *mecA* encoding methicillin resistance, and *ampC* encoding ampicillin resistance in wastewater systems and water biofilms. Also, the application of a multiplex PCR technique has made it easier for monitoring multiple antibiotic resistance genes in various bacterial isolates (Anjum *et al.*, 2017).

Flies captured from different animal rearing facilities have been shown to be vectors for different microorganisms (Zurek *et al.*, 2000). Some of which are foodborne pathogens that pose significant threats to human and global public health (Barreiro *et al.*, 2013; WHO, 2000). Resistance to antibiotics is not limited to the bacteria found in hospital settings but have been increasingly identified in outpatient populations, day-care centres, as well as in people with no known risk factors for acquiring resistant bacteria (Charlebois *et al.*, 2002). Thus, hospice patients are specifically susceptible to bacterial infection because of suppressed immune function following chemotherapeutic regimens or as the result of multiple comorbid conditions, functional debility, and complex diseases that also compromise host resistance (Albrecht *et al.*, 2013). Therefore, this study sought to document antibiotic-resistant and virulence genes from houseflies collected from hospices in Bloemfontein and Johannesburg cities by species-specific PCR assays and Sanger sequencing.

6.2 MATERIALS AND METHODS

6.2.1 Study area, housefly sampling and DNA extraction

Study area, housefly sampling and identification as well as DNA extraction using Qiagen DNeasy PowerSoil Kit (QIAGEN, Hilden, Germany) have been described in detail under section 4.2.1, 4.2.2 and 4.2.3 of Chapter 4 respectively.

6.2.2 Screening for antibiotic resistance, virulence and integrons genes

Antibiotic-resistant genes were selected based on their clinical and veterinary medicine importance (Rodriguez-Mozaz, *et al.*, 2015) and because they are of common concern in the environmental samples (Jiang *et al.*, 2013). The presence of nine antibiotic-resistant genes including β -lactam (*blaCARB*, *blaTEM* and *blaSHV*), tetracycline (*tetA*, *tetW* and *tetX*), and penicillin (*ermB*, *mecA*, and *vanA*) were detected by polymerase chain reaction (PCR) detection assays to determine the presence or absence of the above-mentioned antibiotic-resistant genes in housefly DNA extracts. Virulence genes including *uge*, *magA* and *kfu* associated with pathogenicity of *Klebsiella* spp. and *invA*, *sefA* and *fliC* associated with *Salmonella* spp. pathogenicity were selected. Different primer sets targeting different antimicrobial resistance, virulence genes and class 1 and 2 integrons were synthesized at Inqaba Biotechnical Industries (Pty) Ltd., Pretoria, South Africa based on the published literature (Table 6.1).

6.2.3 PCR for amplification of resistant and virulence genes

All PCR reactions were performed with the final reaction volume of 25 μ l which consisted of one microliter (μ l) of template DNA (± 100 ng/ μ l), 8.5 μ l double distilled water, 2X master mix with standard buffer (OneTaq Quick load buffer, 4 mM MgCl₂, 0.4 mM of each dNTP and 1 unit/ μ l of thermostable *Taq* polymerase (Thermo Scientific, USA), the primer mix contained 1 μ M of each oligonucleotide primer.

The PCR reactions were performed as follows: initial denaturation at 94°C for 6 min, followed by 30 cycles of 94°C for 30 s, annealing temperature (listed in Table 6.1) for

30 s, and 72°C for 60 s, with a final extension at 72°C for 6 min. The presence of resistant and virulence genes was determined by the PCR amplicon band of the expected size in 1% agarose gel electrophoresis in 1× TAE buffer run at 100 V for 30 min. Both negative control (sterile pure water) and positive control (16S rRNA, Table 6.1) according to Jiang *et al.*, (2013) were included in PCR run to ensure the correct operation of PCR reactions.

6.2.4 Purification of amplicons and sequencing

Twenty microliters of all positively amplified PCR products were sent to a commercial sequencing company (Inqaba Biotechnical Industries (Pty) Ltd) for purification and Sanger sequencing in both directions. The quality of resultant sequences was assessed using both MEGA X and Geneious Ver. 7.1 [<http://www.geneious.com>] (Kearse *et al.*, 2012), before consensus sequences were generated from both forward and reverse sequence reads for all generated gene fragments, sequences were edited to remove degenerate base pairs and subsequently trimmed.

6.2.5 Phylogenetic analysis

Generated sequences were edited using MEGAX (Kumar *et al.*, 2018). All edited *blaTEM*, *blaSHV*, *tetA*, *ermB*, *intI* and *intII* gene sequences were subsequently subjected to BLASTn (www.ncbi.nlm.nih.gov/blast/) to confirm their identities. Sequences that belonged to a specific antibiotic-resistant gene were identified based on being the first and closest match from BLASTn results as well as from the identity match score and the lowest E-value. Gene sequences of species closely related to the antibiotic-resistant gene of interest from the BLASTn in search results were subsequently downloaded from the NCBI database for phylogenetic analysis. Successively, all sequences were aligned by ClustalW with default parameters for multiple and pairwise sequence alignments (Kumar *et al.*, 2018). The MEGAX software was used to edit and trim as well as to determine the appropriate evolutionary model to construct the maximum likelihood (ML) tree. The substitution pattern test of homogeneity among sequences was performed to select the finest test model (described below) to compute evolutionary distances among antibiotic-resistant genes

detected. Phylogenetic relationship was determined between antibiotic-resistant gene sequences obtained in this study along with those downloaded sequences from the NCBI database.

The phylogenetic trees for all the resistant genes and classes of integrons were constructed. For the Maximum Likelihood (ML) analysis nodal support was assessed using 1000 rapid bootstrap inferences for all the genes. Moreover, gaps and missing data in the alignments were eliminated from the evolutionary analysis (Tamaru *et al.*, 1992) and the following process as outlined below was applied to construct the ML tree for each gene.

- (i) For *ermB* gene the best model used was the Tamura 3 parameter model (Nei and Kumar, 2000; Kumar *et al.*, 2018). A total of 7 *ermB* gene sequences were used to construct the tree. Sequences of *Streptococcus pyogenes* *mef* and *mefA* genes with accession numbers AF227521 and U70055 respectively, for macrolide-efflux protein A were used as outgroups for rooting the phylogenetic tree.
- (ii) For *tetA* gene, the best model selected was the Tamura 3 parameter model (Nei and Kumar, 2000; Kumar *et al.*, 2018). A total of 14 *tetA* gene sequences were used to construct the tree. The sequence from *Aquifex aeolicus* that code for *fusA* gene (AE000657) for translation elongation factor EF-G was used as the outgroup for rooting the phylogenetic tree.
- (iii) For the *blaSHV* gene, the best model selected was the Tamura 3-parameter (Nei and Kumar, 2000; Kumar *et al.*, 2018). A total of 13 *blaSHV* gene sequences were used to construct the tree. The sequence from cloning vector (MT612433) encoding chromosomal beta-lactamase *blaTEM* resistant gene was used as the outgroup for rooting the phylogenetic tree.
- (iv) (iv) For the *blaTEM* gene, the best model selected was the Jukes-Cantor model (Kumar *et al.*, 2018; Nei and Kumar, 2000). A total of eleven *blaTEM* gene sequences which were used to construct the tree. The sequence of *Klebsiella pneumoniae* (AJ635404) for the chromosomal beta-lactamase (*blaOKP-A* gene) was used as the outgroup for rooting the phylogenetic tree.

- (v) For Class 1 integrons (*IntI*), the best model selected was the Tamura 3 parameter model (Nei and Kumar, 2000; Kumar *et al.*, 2018). There was a total of 9 *IntI* gene sequences which were used to construct the tree. The sequence of *Salmonella typhimurium xerC* gene (U92525) that encode for recombinase of bacteriophage lambda integrase family was used as the outgroup for rooting the phylogenetic tree.
- (vi) For Class 2 integrons (*IntII*), the best model selected was the Kimura 2-parameter model (Nei and Kumar, 2000; Kumar *et al.*, 2018). There was a total of 6 *IntII* gene sequences which were used to construct the tree. The sequence of *Salmonella typhimurium xerC* gene (U92525) that encode for recombinase of bacteriophage lambda integrase family was used as the outgroup for rooting the phylogenetic tree.

6.2.6 Statistical analysis

Data was recorded in Microsoft Excel (2010) spreadsheet for descriptive statistical analysis. The prevalence of antibiotic-resistant genes and 2 classes of integrons from hospices were expressed as percentages based on the total number of houseflies' samples that tested positive for antibiotic-resistant detection from the overall total number of flies tested. Thereafter, one-way ANOVA was used to calculate the significant difference of antibiotic-resistant detection between the hospices. A *P*-value < 0.05 was considered statistically significant.

Table 6.1 Primer sequences used for detection of antimicrobial resistance and virulence genes in housefly DNA extracts

Resistance gene detected	Primer direction	Sequence (5'–3')	Annealing temperature (°C)	Amplicon size (bp)	References
Macrolide antibiotic-resistant genes					
<i>ermB</i>	F	GCATTTAACGACGAACTGGCT	61	573	Jiang <i>et al.</i> , 2013
	R	GACAATACTTGCTCATAAGTAATGGT			
<i>mecA</i>	F	TAATAGTTGTAGTTGTCGGGTTTG	61	733	Jiang <i>et al.</i> , 2013
	R	TAACCTAATAGATGTGAAGTCGCT			
<i>vanA</i>	F	GAAATCAACCATGTTGATGTAGCA	61	572	Jiang <i>et al.</i> , 2013
	R	TTTGCCGTTTCCTGTATCCGT			
β -lactam antibiotic-resistant genes					
<i>blaCARB</i>	F	CAAGTACTTTYAAAAACAATAGC	46	534	Jiang <i>et al.</i> , 2013
	R	GCTGTAATACTCCKAGCAC			
<i>blaTEM</i>	F	AAAGATGCTGAAGATCA	44	425	Jiang <i>et al.</i> , 2013
	R	TTTGGTATGGCTTCATTC			
<i>blaSHV</i>	F	GCGAAAGCCAGCTGTCGGGC	62	304	Jiang <i>et al.</i> , 2013
	R	GATTGGCGGCGCTGTTATCGC			
Tetracycline antibiotic-resistant genes					
<i>tetA</i>	F	GCGCTNTATGCGTTGATGCA	62	387	Jiang <i>et al.</i> , 2013
	R	ACAGCCCGTCAGGAAATT			
<i>tetW</i>	F	GAGAGCCTGCTATATGCCAGC		168	Jiang <i>et al.</i> , 2013
	R	GGGCGTATCCACAATGTTAAC	64		
<i>tetX</i>	F	CCGACACGGAAGTTGAAGAA		468	Jiang <i>et al.</i> , 2013
	R	CCTTGGTGAGATGCCATTAGC	60		
Integrans					
<i>intl</i>	F	GATGCCTGCTTGTTCTAC	55	558	Ng <i>et al.</i> , 1999
	R	GCCTTGCTGTTCTTCTAC			
<i>intlI</i>	F	GTAGCAAACGAGTGACGAAATG	62	788	Peerayeh and Karmastaji 2015
	R	CACGGATATGCGACAAAAGGT			
16S <i>rRNA</i>		16S <i>rRNA</i>			Mignard and Flandrois, 2006
	F	GGAATTCAAAGGAATTGACGGGGGC		440	
	R	CGGGATCCCAGGCCCGGAACGTATTAC	-		

Virulence genes					
<i>invA</i>	invA 139	GTGAAATTATCGCCACGTTTCGGGCAA	53	284	Bisi-Johnson <i>et al.</i> , 2011
	invA 141	TCATCGCACCGTCAAAGGAACC			
<i>sefA</i>	S1	GCCGTACACGAGCTTATAGA	53	250	Bisi-Johnson <i>et al.</i> , 2011
	S4	ACCTACAGGGGCACAATAAC			
<i>fliC</i>	Fli15	CGGTGTTGCCCAGGTTGGTAAT	53	620	Bisi-Johnson <i>et al.</i> , 2011
	Typ04	ACTGGTAAAGATGGCT			
<i>uge</i>	F	TCT TCA CGC CTT CCT TCA CT	54	534	Aher <i>et al.</i> , 2012
	R	GAT CAT CCG GTC TCC CTG TA			
<i>magA</i>	F	GGT GCT CTT TAC ATC ATT GC	59	1280	Aher <i>et al.</i> , 2012
	R	GCA ATG GCC ATT TGC GTT AG			
<i>kfu</i>	F	GAA GTG ACG CTG TTT CTG GC	54	797	Aher <i>et al.</i> , 2012
	R	TTT CGT GTG GCC AGT GAC TC			

6.3 RESULTS

6.3.1 Virulence genes

None of the virulence genes associated with pathogenicity of *Klebsiella* spp. and *Salmonella* spp. were detected by PCR assays used in this study.

6.3.2 Antibiotic-resistant genes

A total of nine (9)-targeted antibiotic-resistant genes and two of the integrons (Table 6.1) were selected in this study and detected by conventional PCR. Thus, only 44.4% (4/9) antibiotic-resistant genes including *ermB*, *tetA*, *blaSHV* and *blaTEM* were detected in the housefly samples from the hospices as summarized in Table 6.2. However, 55.6% (5/9) antibiotic-resistant genes including *blaCARB*, *van*, *tetX*, *tetW* and *mecA* were not detected. Successfully amplified resistant genes are shown in figures 6.1 - 6.3 with *ermB*, *tetA*, *blaSHV* and *blaTEM* PCR positive at 573 bp, 387 bp, 304 bp and 425 bp, respectively. The integrons *intI* and *intII* were also positively detected at 558 bp and 788 bp respectively (Figure 6.4 and 6.5).

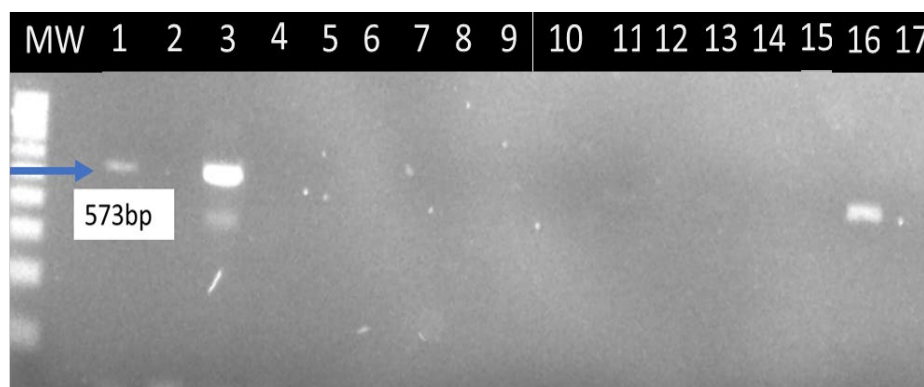


Figure 6.1 Detection of the 573 bp *ermB* gene amplicon from representative housefly DNA extracts. MW is the molecular weight marker, lane 1–17 test samples.

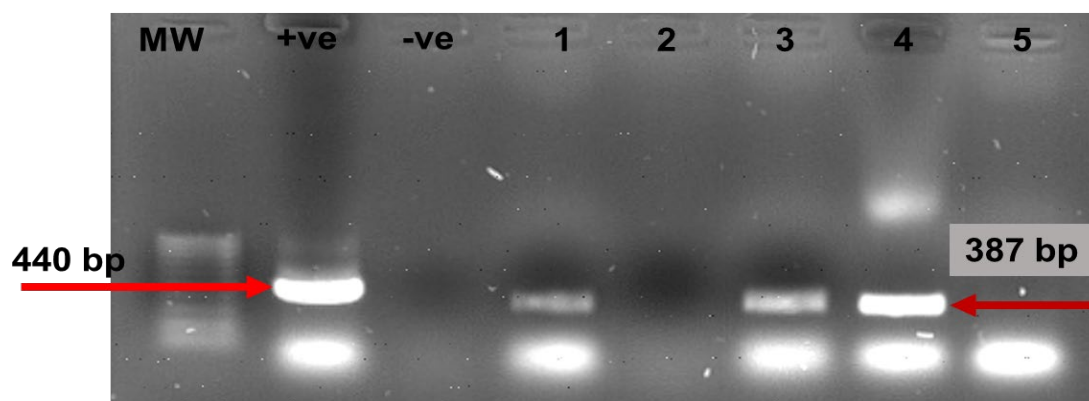


Figure 6.2 Detection of the 387bp *tetA* gene amplicon from representative housefly DNA extracts. MW is the molecular weight marker, -ve is for the no template control, +ve is the 16S *rRNA* positive control. Lane 1,3 and 4 show samples positive for presence of *tetA* gene from hospices samples whilst Lane 2 and 5 show samples negative for absence of *tetA* gene.

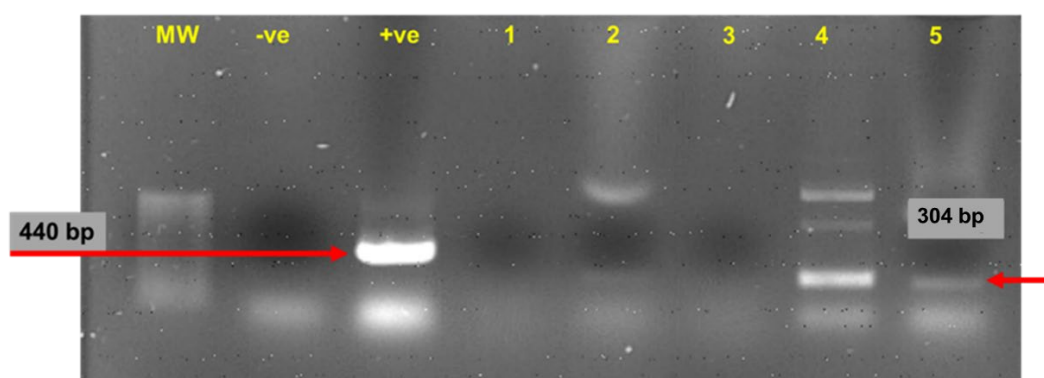


Figure 6.3. Detection of the 304 bp *blaSHV* gene amplicon from representative housefly DNA extracts. MW is the molecular weight marker, -ve is for the no template control, +ve is the 16S *rRNA* positive control. Lane 2, 4 and 5 show samples positive for the presence of *blaSHV* gene from hospices samples, however, lane 1 and 3 shows the absence of *blaSHV* gene in the samples.

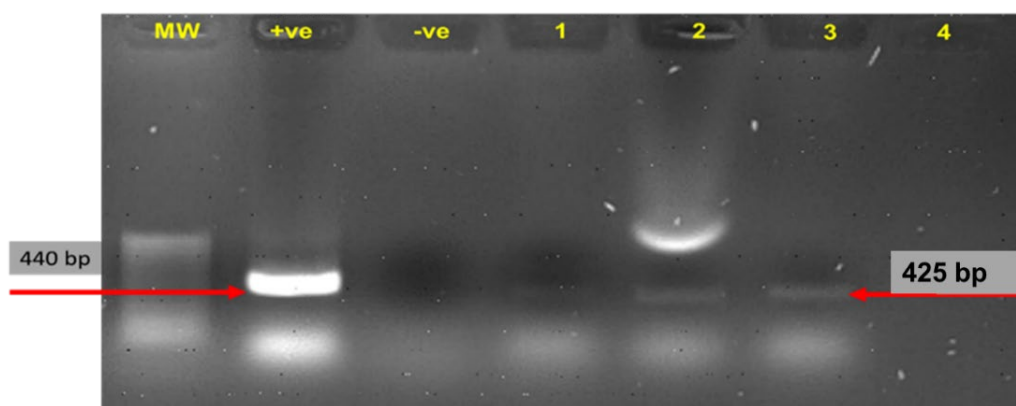


Figure 6.4 Detection of the 425 bp *blaTEM* gene amplicon from representative housefly DNA extracts. MW is the molecular weight marker, -ve is for the no template control, +ve is the 16S *rRNA* positive control. Lane 1, 2, and 3 show samples positive for the presence of *blaTEM* gene in the samples, however, lane 4 shows the absence of *blaTEM* gene in the samples.

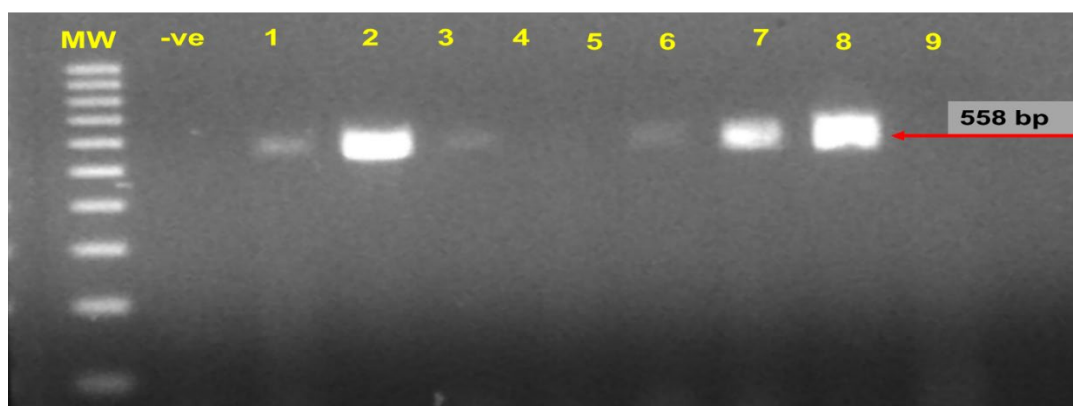


Figure 6.5 Detection of the 565 bp *nt11* gene amplicon from representative housefly DNA extracts. MW is the molecular weight marker, -ve is for the no template control. Lane M 100 bp marker; lane 1–9 shows test samples.

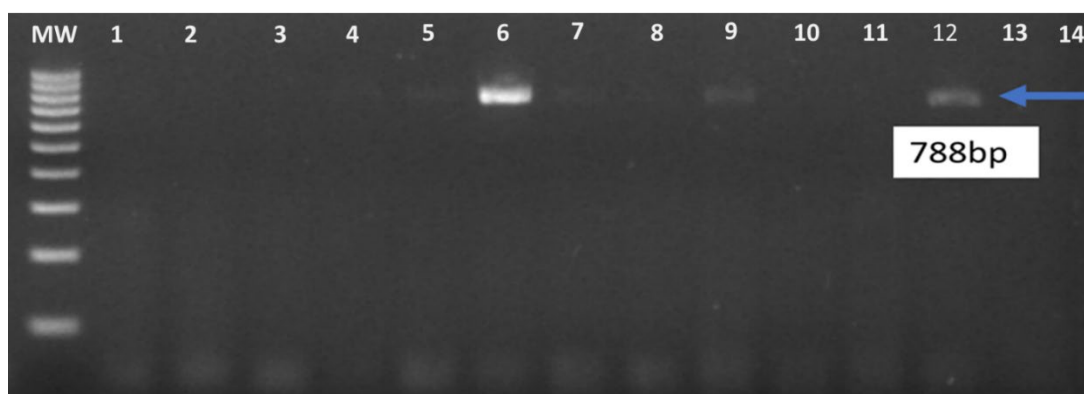


Figure 6.6 Detection of the 788 bp *IntI* gene amplicon from representative housefly DNA extracts. MW is the molecular weight marker, -ve is for the no template control. Lane M 100 bp marker, lane 1–14 test samples.

6.3.3 Occurrence of antibiotic-resistant genes

6.3.3.1 β -lactam resistance gene

Antibiotic-resistant genes, *blaTEM* and *blaSHV* were the only detected β -lactam resistance genes. Antibiotic-resistant gene *blaTEM* was present in 26.7% of the samples from Lebone hospice and 13.3 % of the samples from Sunflower hospice. No *blaCARB* and *ampC* were detected in any of the sample obtained from all three sampled hospices. No β -lactam antibiotic-resistant genes were detected from samples obtained from Sparrow hospice.

6.3.3.2 Macrolide resistant genes

Antibiotic-resistant gene, *ermB* was the only detected penicillin-resistant gene from both Lebone hospice and Sparrow hospice with the detection frequency of 13.3% and 6.6%, respectively. None of *mecA* and *vanA* genes was detected in samples obtained from all three sampled hospices. No penicillin antibiotic-resistant gene was detected from samples obtained from Sunflower hospice.

6.3.3.3 Tetracycline resistance genes

Antibiotic-resistant genes *tetA* was detected from housefly DNA extracts samples obtained from all sampled hospices with the detection frequency of 60% from Lebone, 33.3% from Sunflower hospice and 20% from Sparrow hospice. Additionally, no *tetX* and *tetW* resistant genes were detected from the samples obtained from all three sampled hospices.

6.3.3.4 Integrations

Class1 and class 2 integrations integrase genes were detected in housefly DNA extract samples obtained from all sampled hospices. The detection frequency of Class1 integrations from Lebone hospice, Sparrow hospice and Sunflower hospice was 53.3%, 40.0% and 46.7%, respectively. The detection frequency of Class 2 integrations from Lebone, Sparrow and Sunflower hospices was 60.0%, 13.3% and 53.3%, respectively.

Table 6.2 Summary of resistant genes determined using conventional polymerase chain reaction (PCR)

Resistant gene detected	Lebone Hospice n=15 (%)	Sparrow Hospice n=15 (%)	Sunflower Hospice n=15 (%)	<i>p-value</i>
Macrolide antibiotic-resistant genes				0.231
<i>ermB</i>	2 (13.3%)	1 (6.6%)	0 (0%)	
<i>mecA</i>	0 (0%)	0 (0%)	0 (0%)	
<i>vanA</i>	0 (0%)	0 (0%)	0 (0%)	
β -lactam antibiotic resistant genes				0.125
<i>blaCARB</i>	0 (0%)	0 (0%)	0 (0%)	
<i>blaTEM</i>	4 (26.7%)	0 (0%)	2 (13.3%)	
<i>blaSHV</i>	6 (40.0%)	0 (0%)	5 (33.3%)	
<i>ampC</i>	0 (0%)	0 (0%)	0 (0%)	
Tetracycline antibiotic-resistant genes				0.122
<i>tetA</i>	9 (60%)	3 (20.0%)	5 (33.3%)	
<i>tetW</i>	0 (0%)	0 (0%)	0(0%)	
<i>tetX</i>	0 (0%)	0 (0%)	0 (0%)	
Integrans				0.783
<i>intl</i>	8 (53.3%)	6 (40.0%)	7 (46.7%)	
<i>intlI</i>	9 (60.0%)	2 (13.3%)	8 (53.3%)	

6.3.4 Comparative analysis

The determined nucleotide sequences in this study were confirmed to correspond with *tetA*, *blaSHV*, *blaTEM*, *ermB*, *intl* and *intlI* gene sequences published in GenBank's NCBI database. The BLASTn homology search in GenBank showed that *tetA* and *blaSHV* gene sequences determined in the present study exhibited 100% nucleotide identity with previously published sequences of the same genes (*tetA* and *blaSHV*), from other bacterial strains.

The alignment of *tetA* and *blaSHV* sequences generated from this study against published sequences from GenBank, showed no gaps nor mutations as shown in Figures 6.7 and 6.8 respectively. On the other hand, *blaTEM* resistant gene sequences

determined in this study exhibited identities of 99% when compared to other published sequences. The alignment of the determined *blaTEM* antibiotic-resistant gene sequence with a published sequence (accession no: MW353735) is shown in figure 6.9 with four gaps with no mutation observed. The *ermB* resistant gene sequences determined in this study exhibited identities of 99% when compared to other published sequences. The alignment of the determined *ermB* antibiotic-resistant gene sequence with a published sequence (accession no: MT050496) is shown in figure 6.10 with one gap and one transversion mutation observed.

For the class 1 Integron (*intI*) gene, the sequences determined in this study exhibited identities of 99% when compared to other published sequences (Table 6.7). The alignment of the determined *intI* gene sequence with a published sequence (accession no: KT317076) in figure 6.11 shown neither gap nor mutation. However, for class 2 Integron gene with the sequence determined in this study exhibited identities of 93% when compared to other published sequences (Table 6.8). The alignment of the determined *intII* gene sequence with a published sequence (accession no: KT317076) in figure 6.12 shows the observation of 13 gaps with 19 mutations (i.e., 7 transition mutations and 12 transversion mutation).

Score	Expect	Identities	Gaps	Strand
715 bits(387)	0.0	387/387(100%)	0/387(0%)	Plus/Plus
Query 2	GCGCTGTATGCGTTGATGCAATTTGCTGCGCACCTGTGCTGGGCGCGCTGTGCGGATCGT	61		
Sbjct 51778	GCGCTGTATGCGTTGATGCAATTTGCTGCGCACCTGTGCTGGGCGCGCTGTGCGGATCGT	51837		
Query 62	TTCGGGCGGCGGCCGGTCTTGCTCGTCTCGCTGGCGGCGCTGCTGTGCGACTACGCCATC	121		
Sbjct 51838	TTCGGGCGGCGGCCGGTCTTGCTCGTCTCGCTGGCGGCGCTGCTGTGCGACTACGCCATC	51897		
Query 122	ATGGCGACGGCGCCTTTCTTTGGGTTCTCTATATCGGGCGGATCGTGGCGGCGATCACC	181		
Sbjct 51898	ATGGCGACGGCGCCTTTCTTTGGGTTCTCTATATCGGGCGGATCGTGGCGGCGATCACC	51957		
Query 182	GGGGCGACTGGGGCGGTAGCCGGCGCTTATATTGCCGATATCACTGATGGCGATGAGCGC	241		
Sbjct 51958	GGGGCGACTGGGGCGGTAGCCGGCGCTTATATTGCCGATATCACTGATGGCGATGAGCGC	52017		
Query 242	GCGCGGCACTTCGGCTTCATGAGCGCCTGTTTCGGGTTTCGGGATGGTCGCGGGACCTGTG	301		
Sbjct 52018	GCGCGGCACTTCGGCTTCATGAGCGCCTGTTTCGGGTTTCGGGATGGTCGCGGGACCTGTG	52077		
Query 302	CTCGGTGGGCTGATGGGCGGTTTCTCCCCACGCTCCGTTCTTCGCCGCGGCGAGCCTTG	361		
Sbjct 52078	CTCGGTGGGCTGATGGGCGGTTTCTCCCCACGCTCCGTTCTTCGCCGCGGCGAGCCTTG	52137		
Query 362	AACGGCCTCAATTTCTGACGGGCTGT	388		
Sbjct 52138	AACGGCCTCAATTTCTGACGGGCTGT	52164		

Figure 6.7 BLASTn results showing the alignment of *tetA* resistant gene and one of the sequences from this study which was from a housefly sample from hospice. The subject sequence accession no: CP063836 covered 99% of the query sequence and it had 100% identity.

Score	Expect	Identities	Gaps	Strand
560 bits(303)	2e-155	303/303(100%)	0/303(0%)	Plus/Minus
Features: class A broad-spectrum beta-lactamase SHV-11				
Query 2	GATTGGCGGCGCTGTTATCGCTCATGGTAATGGCGGCGGCACAGAGTTCGCCGACCGTCA	61		
Sbjct 2838720	GATTGGCGGCGCTGTTATCGCTCATGGTAATGGCGGCGGCACAGAGTTCGCCGACCGTCA	283866		
Query 62	TGCCGTCGGCAAGGTGTTTTTCGCTGACCGGCGAGTAGTCCACCAGATCCTGCTGGCGAT	121		
Sbjct 2838660	TGCCGTCGGCAAGGTGTTTTTCGCTGACCGGCGAGTAGTCCACCAGATCCTGCTGGCGAT	283866		
Query 122	AGTGGATCTTTCGCTCCAGCTGTTTCGTCACCGGCATCCACCCGCCAGCACTGCGCCGC	181		
Sbjct 2838600	AGTGGATCTTTCGCTCCAGCTGTTTCGTCACCGGCATCCACCCGCCAGCACTGCGCCGC	283854		
Query 182	AGAGCACTACTTTAAAGGTGCTCATCATGGGAAAGCGTTTCATCGGCGGCCAGGCGGTCA	241		
Sbjct 2838540	AGAGCACTACTTTAAAGGTGCTCATCATGGGAAAGCGTTTCATCGGCGGCCAGGCGGTCA	283848		
Query 242	GCGTGCGGCGCTGGCCAGATCCATTTCTATCATGCCTACGCGGCCGACAGCTGGCTTT	301		
Sbjct 2838480	GCGTGCGGCGCTGGCCAGATCCATTTCTATCATGCCTACGCGGCCGACAGCTGGCTTT	283842		
Query 302	CGC	304		
Sbjct 2838420	CGC	2838418		

Figure 6.8. BLASTn results showing the alignment of the *blaSHV* resistant gene and one of the sequences from this study which was from a housefly sample from hospice. The subject sequence accession no: CP052136 covered 99% of the query sequence and it had 100% identity.

Score	Expect	Identities	Gaps	Strand
767 bits(415)	0.0	425/429(99%)	4/429(0%)	Plus/Plus
Query 245	AAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAAGCAG	304		
Sbjct 8859	AAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAAGCAG	8917		
Query 305	CGGTACAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTA	364		
Sbjct 8918	CGGTACAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTTTA	8976		
Query 365	AAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTC	424		
Sbjct 8977	AAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGTC	9036		
Query 425	GCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTACCAAGTCACAGAAAAGCATC	484		
Sbjct 9037	GCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTACCAAGTCACAGAAAAGCATC	9096		
Query 485	TTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACA	544		
Sbjct 9097	TTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACA	9156		
Query 545	CTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGC	604		
Sbjct 9157	CTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGC	9216		
Query 605	ACAACATGGGGTATCACTGTAACTCGCCTTGATCGTTGGGAACCGAGCTGAATGAAGC	664		
Sbjct 9217	ACAACATGGGGTATCACTGTAACTCGCCTTGATCGTTGGGAACCGAGCTGAATGAAGC	9274		
Query 665	CATACCAA 673			
Sbjct 9275	CATACCAA 9283			

Figure 6.9 BLASTn results showing the alignment of the *blaTEM* resistant gene and one of the sequences from this study which was from a housefly sample from hospice. The subject sequence accession no: MW353735 with 99% identity with four gaps and no mutation.

Score	Expect	Identities	Gaps	Strand
1048 bits(567)	0.0	574/577(99%)	1/577(0%)	Plus/Plus
Query 2	GCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAG	61		
Sbjct 123	GCATTTAACGACGAAACTGGCTAAAATAAGTAAACAGGTAACGTCTATTGAATTAGACAG	182		
Query 62	TCATCTATTCAACTTATCGTCAGAAAAATTAATAACTGAA*ATTCGTGTCACTTTAATTCA	121		
Sbjct 183	TCATCTATTCAACTTATCGTCAGAAAAATTAATAACTGAACATTCGTGTCACTTTAATTCA	242		
Query 122	CCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAATAT	181		
Sbjct 243	CCAAGATATTCTACAGTTTCAATTCCCTAACAAACAGAGGTATAAAATTGTTGGGAATAT	302		
Query 182	TCCTTACCATTTAAGCACACAAATTATTAATAAAAGTGGTTTTTGAAAGCCATGCGTCTGA	241		
Sbjct 303	TCCTTACCATTTAAGCACACAAATTATTAATAAAAGTGGTTTTTGAAAGCCATGCGTCTGA	362		
Query 242	CATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTACCGAACACT	301		
Sbjct 363	CATCTATCTGATTGTTGAAGAAGGATTCTACAAGCGTACCTTGGATATTACCGAACACT	422		
Query 302	AGGGTTGCTCTTGACACTCAAGTCTCGATTACGCAATTGCTTAAGCTGCCAGCGGAATG	361		
Sbjct 423	AGGGTTGCTCTTGACACTCAAGTCTCGATTACGCAATTGCTTAAGCTGCCAGCGGAATG	482		
Query 362	CTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCACAGA	421		
Sbjct 483	CTTTCATCCTAAACCAAAAGTAAACAGTGTCTTAATAAAACTTACCCGCCATACCACAGA	542		
Query 422	TGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGA	481		
Sbjct 543	TGTTCCAGATAAATATTGGAAGCTATATACGTACTTTGTTTCAAAATGGGTCAATCGAGA	602		
Query 482	ATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCAATGAAACACTGCCAAAGTAA	541		
Sbjct 603	ATATCGTCAACTGTTTACTAAAAATCAGTTTCATCAAGCAATGAAACAC-GCCAAAGTAA	661		
Query 542	ACAATTTAAGTACC*ATTACTTATGAGCAAGTATTGTC	578		
Sbjct 662	ACAATTTAAGTACCGTTACTTATGAGCAAGTATTGTC	698		

Figure 6.10 BLASTn results showing the alignment of *erm(B)* resistant gene and one of the sequences from this study which was from a housefly sample from hospice. The subject sequence accession no: MT050496 covered 99% of the query sequence and it had 99% identity with one gap and two mutations shown by asterisk.

Score	Expect	Identities	Gaps	Strand
957 bits(518)	0.0	518/518(100%)	0/518(0%)	Plus/Minus
Query 2	GCCTTGCTGTTCTTTTACGGCAAGGTGCTGTGCACGGATCTGCCCTGGCTTCAGGAGATC	61		
Sbjct 3407	GCCTTGCTGTTCTTTTACGGCAAGGTGCTGTGCACGGATCTGCCCTGGCTTCAGGAGATC	3348		
Query 62	GGAAGACCTCGGCCGTCGCGGCGCTTGCCGGTGGTGCTGACCCCGGATGAAGTGGTTTCGC	121		
Sbjct 3347	GGAAGACCTCGGCCGTCGCGGCGCTTGCCGGTGGTGCTGACCCCGGATGAAGTGGTTTCGC	3288		
Query 122	ATCCTCGGTTTTCTGGAAGGCGAGCATCGTTTGTTGCGCCAGCTTCTGTATGGAACGGGC	181		
Sbjct 3287	ATCCTCGGTTTTCTGGAAGGCGAGCATCGTTTGTTGCGCCAGCTTCTGTATGGAACGGGC	3228		
Query 182	ATGCGGATCAGTGAGGGTTTGCAACTGCGGGTCAAGGATCTGGATTTTCGATCACGGCACG	241		
Sbjct 3227	ATGCGGATCAGTGAGGGTTTGCAACTGCGGGTCAAGGATCTGGATTTTCGATCACGGCACG	3168		
Query 242	ATCATCGTGCGGGAGGGCAAGGGCTCAAGGATCGGGCCTTGATGTTACCCGAGAGCTTG	301		
Sbjct 3167	ATCATCGTGCGGGAGGGCAAGGGCTCAAGGATCGGGCCTTGATGTTACCCGAGAGCTTG	3108		
Query 302	GCACCCAGCCTGCGCGAGCAGCTGTGCGTGCACGGGCATGGTGGCTGAAGGACCAGGCC	361		
Sbjct 3107	GCACCCAGCCTGCGCGAGCAGCTGTGCGTGCACGGGCATGGTGGCTGAAGGACCAGGCC	3048		
Query 362	GAGGGCCGCGAGCGGCGTTGCGCTTCCCGACGCCCTTGAGCGGAAGTATCCGCGCGCCGGG	421		
Sbjct 3047	GAGGGCCGCGAGCGGCGTTGCGCTTCCCGACGCCCTTGAGCGGAAGTATCCGCGCGCCGGG	2988		
Query 422	CATTCTGGCCGTGGTTCTGGGTTTTTGCGCAGCACACGCATTGACCGATCCACGGAGC	481		
Sbjct 2987	CATTCTGGCCGTGGTTCTGGGTTTTTGCGCAGCACACGCATTGACCGATCCACGGAGC	2928		
Query 482	GGTGTCGTGCGTCGCCATCACATGTATGACCAGACCTT	519		
Sbjct 2927	GGTGTCGTGCGTCGCCATCACATGTATGACCAGACCTT	2890		

Figure 6.11 BLASTn results showing the alignment of *intl* gene and one of the sequences from this study which was from a housefly DNA sample from hospice. The subject sequence accession no: KT317076 covered 92% of the query sequence and it had 100% identity.

Score	Expect	Identities	Gaps	Strand
846 bits(458)	0.0	541/579(93%)	13/579(2%)	Plus/Minus
Query 72	GCAGATGACGGGGGCATAATTTGCCGTTATACGGGTGGTTGCGGAGCGTGCTGGAGGGAA	131		
Sbjct 626	GCAGATGATGGCGGCATAATTTGCCGTTATACGGGTGGTTGCAGAGCGTGCTGGAGGGAA	567		
Query 132	AGACAAACATCCACGCCGCTTGTGCGATAAGCAGAAG---ATTGTGATCTAAAGCAAAAG	188		
Sbjct 566	AGACAAACATCCACGCCGCTTGTGCGATAAGCAGAAGGGTATTGTGATCTAAAGCAAAAG	507		
Query 189	GCAGCGATGGCCCTACGCCTTGTAAGTTG---CGTCTTGCTGAATA---GCCGCGCTTGCTCAA	246		
Sbjct 506	GCAGCGATGGCCCTACGCCTTGTAAGTTGTCGTCTTGCTGAATAAGCCGCGCTTGCTCAA	447		
Query 247	TGAGTTATTTTATTGCTGGGATTAGGCGCGTGGGCAGTAGGCTGTTTCTGCTTTTCCAC	306		
Sbjct 446	TGAGTTATTTTATTGCTGGGATTAGGCGCGTGGGCAGTAGGCTGTTTCTGCTTTTCCAC	387		
Query 307	CCTTACCGTCATGCACAGTGATGCA---CCATTATCAA---CAAAATCTTTAACC CGCAAAC	362		
Sbjct 386	CCTTACCGTCATGCACAGTGATGCA---CCATTATCAA---CAAAATCTTTAACC CGCAAAC	327		
Query 363	GCAAGCATTCAATTAATGCGCAAACCTGCACCATACAGCAGCGTAAAAATAACTTGGTTGC	422		
Sbjct 326	GCAAGCATTCAATTAATGCGCAAACCTGCACCATACAGCAGCGTAAAAATAACTTGGTTGC	267		
Query 423	GAGTATCCATAACCTGCAAAATGCGTTGCACTTCATTTGCAGAGATAACAGAGGGTAGCC	482		
Sbjct 266	GAGTATCCATAACCTGCAAAATGCGTTGCACTTCATTTGCAGAGATAACAGAGGGTAGCC	207		
Query 483	GTCTAGGCTTGCTTG---AGGGATATAATCAATATCGCCCAACGGCTGGTGGAAAAACCTGT	541		
Sbjct 206	GTCTAGGCTTGCTTG---AGGGATATAATCAATATCGCCCAACGGCTGTTGTAAAAACCTGT	147		
Query 542	TGGACAAGAAATCTAGGGCGTTGAAAG---GATTTTCTGCGT*****CTAGGTGTCTGG	600		
Sbjct 146	TGTACAAAAAAGCTAGGGCATTAAAGCGATTTTCTGCGTGTTATGGCTACATGTCTGC	87		
Query 601	TGGTTGCTAGGG---GGAGAAG---ACGGACTGACCTCTTCAC	637		
Sbjct 86	TGTTTGCTAAGCTGGATAAAACAGCCTGACCTCTTCAC	48		

Figure 6.12 BLASTn results showing the alignment of *intlI* resistant gene and one of the sequences from this study which was from a housefly sample from hospice. The subject sequence accession no: MT700997 covered 76% of the query sequence and it had 93% identity with 13 gaps and 19 mutations shown by the asterisk.

6.3.5 Phylogenetic analysis

The maximum likelihood trees with 1000 bootstraps replicate values expressed as percentages were constructed with *tetA*, *blaSHV*, *blaTEM*, *ermB*, *intlI* and *intlII* gene sequences detected from housefly microbiota in this study. Closely related sequences of all the detected antibiotic-resistant genes and classes of integrons retrieved from GenBank were also included in the phylogeny reconstruction. The respective cladograms

are shown in figure 6.13-6.17. All the resistance genes sequences obtained in this study shown to be closely related with other resistance genes sequences obtained from NCBI. The genes were found to be in various bacterial species and different cloning vectors.



Figure 6.13 Phylogenetic analysis of *tetA* resistant gene sequences detected from the housefly microbiota DNA samples obtained from the hospices. The tree was constructed using the maximum likelihood method. Numbers above each node show the percentage of the tree configurations that occurred during the 1000 bootstrap trials. The horizontal bar represents the number of substitutions per sites. The sequence of the *Aquifex aeolicus* fusA gene (AE000657) for translation elongation factor EF-G was used as an outgroup for rooting the phylogenetic tree.

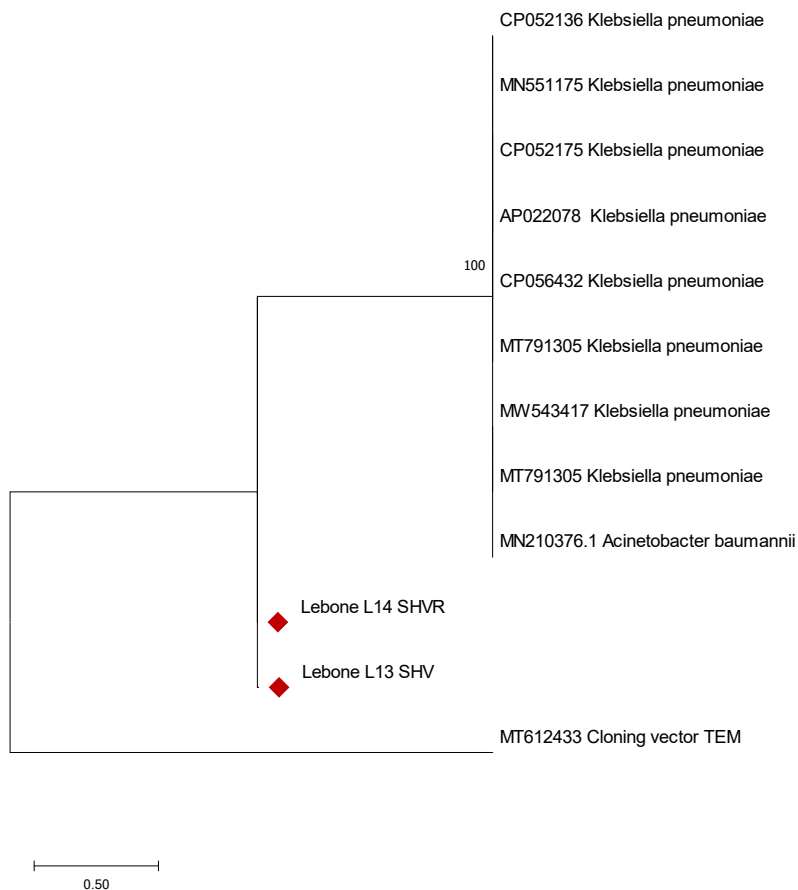


Figure 6.14 Phylogenetic analysis of *blaSHV* resistant gene sequences detected from the housefly microbiota DNA samples obtained from the hospices. The tree was constructed using the maximum likelihood method. Numbers above each node show the percentage of the tree configurations that occurred during the 1000 bootstrap trials. The horizontal bar represents the number of substitutions per sites. The sequence from the cloning vector (MT612433) encoding the chromosomal beta-lactamase *blaTEM* resistant gene was used as an outgroup for rooting the phylogenetic tree.

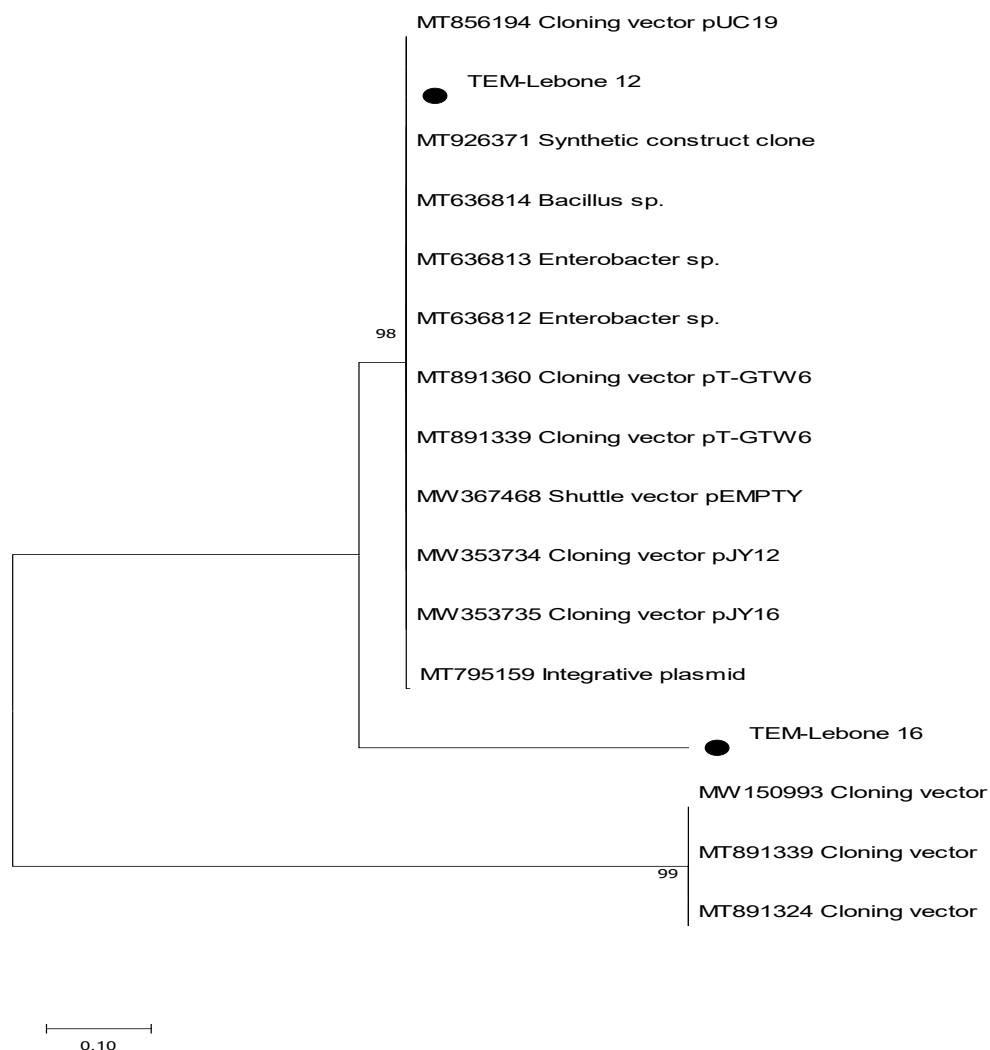


Figure 6.15 Phylogenetic analysis of *bla*_{TEM} resistant gene sequences detected from the housefly microbiota DNA samples obtained from the hospices. The tree was constructed using the maximum likelihood method. Numbers above each node show the percentage of the tree configurations that occurred during the 1000 bootstrap trials. The horizontal bar represents the number of substitutions per sites. The sequence of the Cloning vector (MW150993, MT891339 and MT891234) for the plasmid-encoded ampicillin resistance gene was used as an outgroup for rooting the phylogenetic tree.

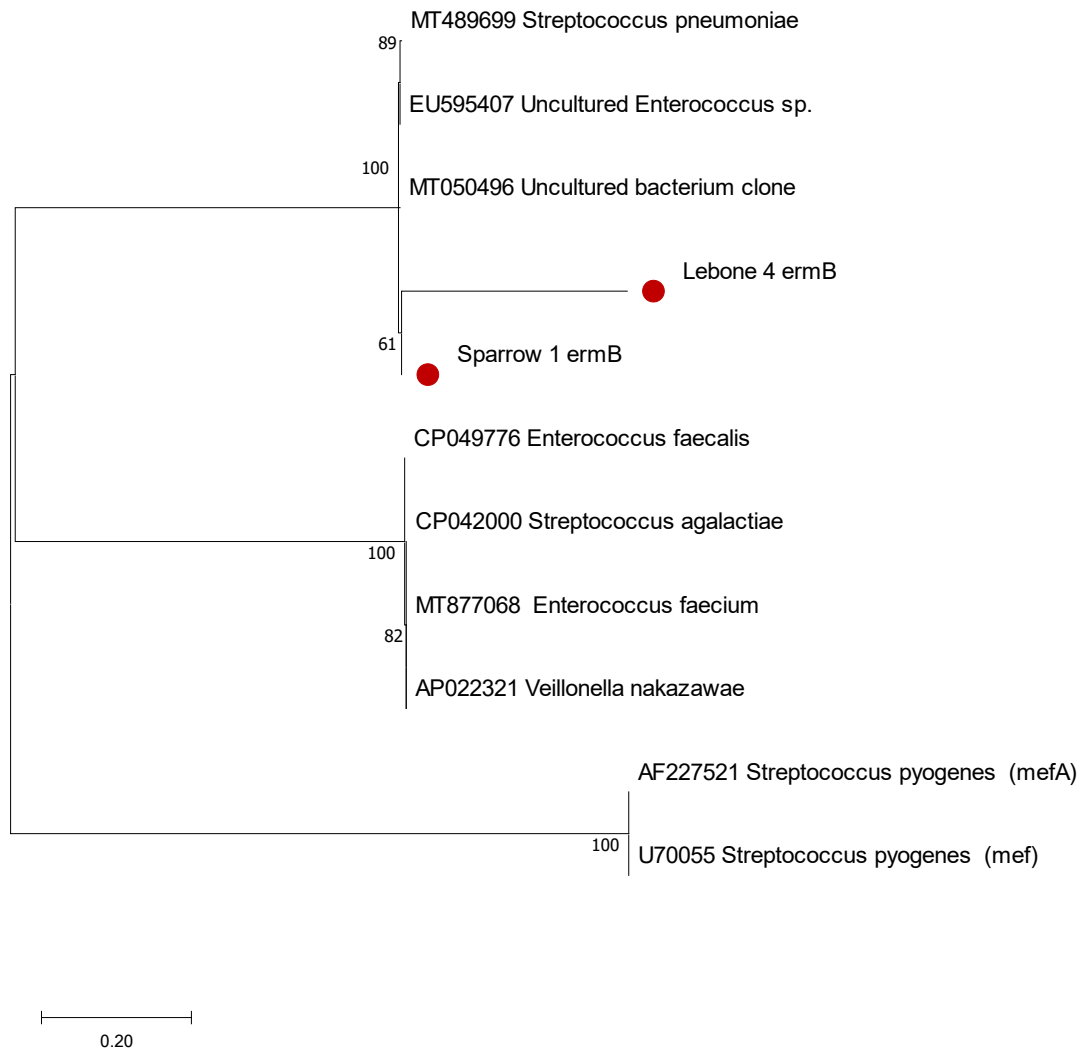


Figure 6.16 Phylogenetic analysis of *ermB* resistant gene sequences detected from the housefly microbiota DNA samples obtained from the hospices. The tree was constructed using the maximum likelihood method. Numbers above each node show the percentage of the tree configurations that occurred during the 1000 bootstrap trials. The horizontal bar represents the number of substitutions per sites. The sequence of the *streptococcus pyogenes mef* and *mefA* gene with accession number Af227521 and U70055 respectively, for macrolide-efflux protein A were used as an outgroup for rooting the phylogenetic tree.

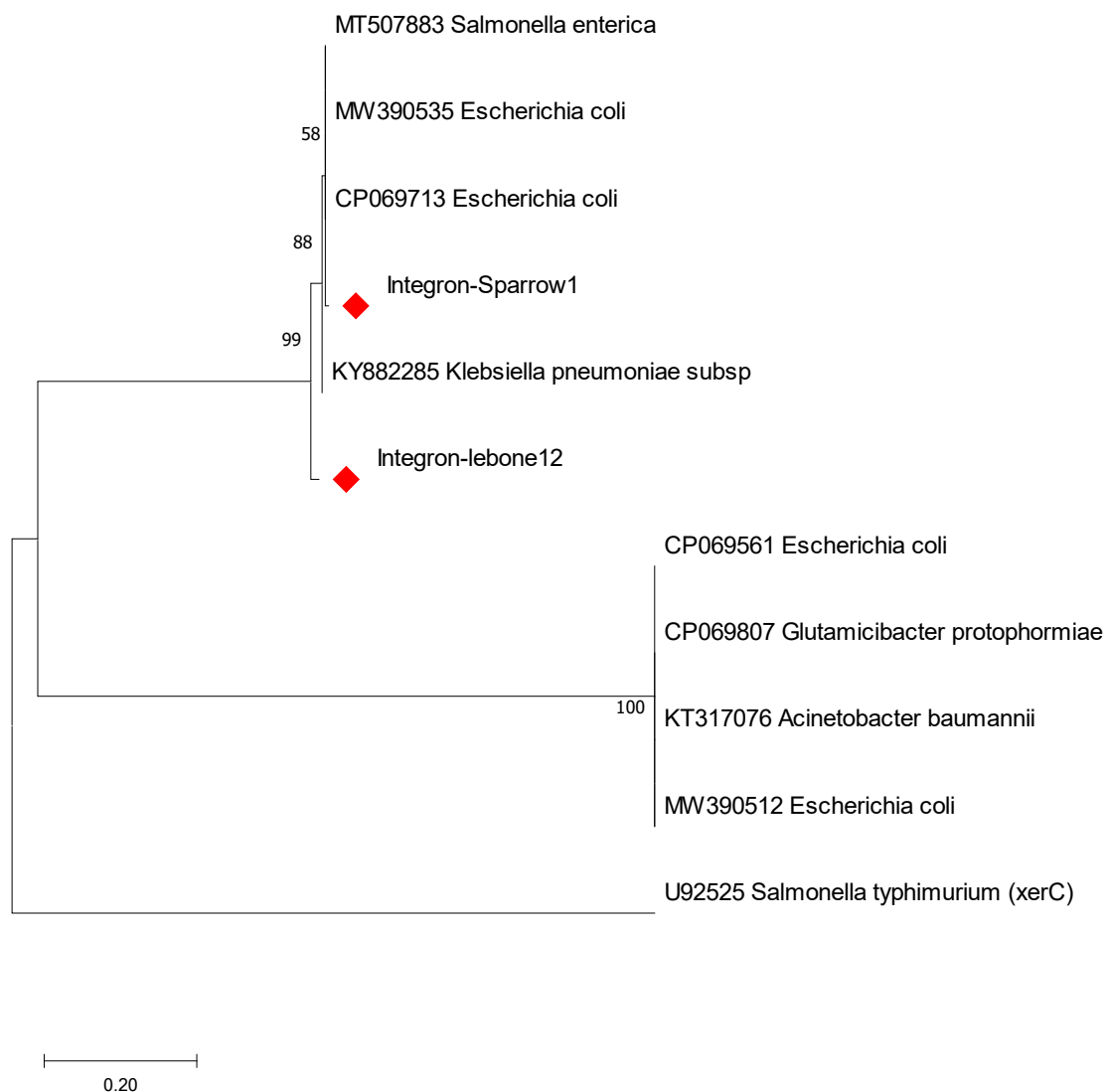


Figure 6.17 Phylogenetic analysis of *intI* sequences detected from the housefly microbiota DNA samples obtained from the hospices. The tree was constructed using the maximum likelihood method. Numbers above each node show the percentage of the tree configurations that occurred during the 1000 bootstrap trials. The horizontal bar represents the number of substitutions per sites. The sequence of the *Salmonella typhimurium xerC* gene (U92525) was used as an outgroup for rooting the phylogenetic tree.

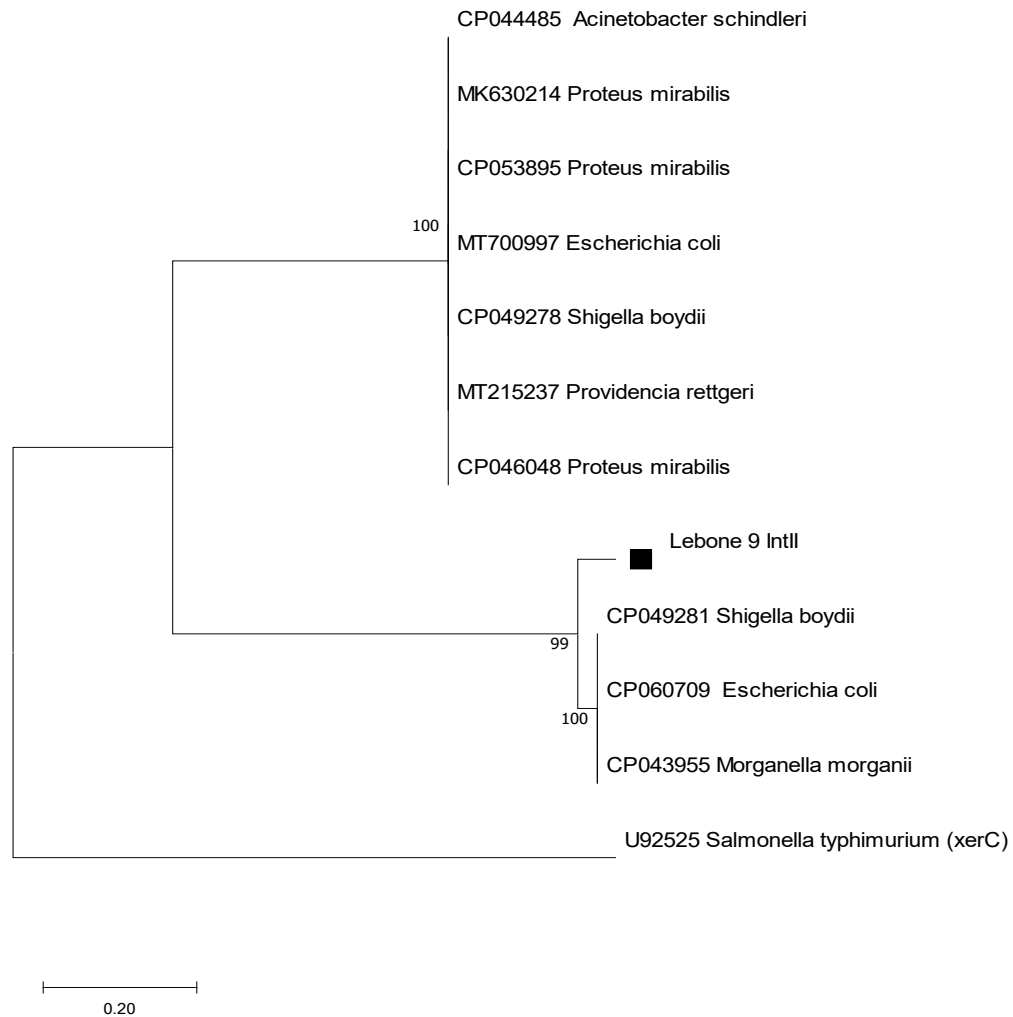


Figure 6.18 Phylogenetic analysis of *intII* sequences detected from the housefly microbiota DNA samples obtained from the hospices. The tree was constructed using the maximum likelihood method. Numbers above each node show the percentage of the tree configurations that occurred during the 1000 bootstrap trials. The horizontal bar represents the number of substitutions per sites. The sequence of the *Salmonella typhimurium xerC* gene (U92525) was used as an outgroup for rooting the phylogenetic tree.

6.4 DISCUSSION

In this study, the occurrence of antibiotic resistance genes in housefly DNA from three hospices situated in Bloemfontein and Johannesburg were investigated. The samples from the hospices were compared among each other. The highest prevalence of antibiotic resistance genes detected in this study was observed from Bloemfontein in Lebone hospice, followed by Sunflower hospice with Sparrow hospice based in Johannesburg being the least. The reason for these occurrences might be due to the agricultural activities including nursery which produces seedlings for vegetable production, raising of broiler chicks and piggery occurring around Lebone hospice area. Whilst, Sunflower hospice is situated next to the hospital. This type of agricultural activities involves the use of antibiotics as prophylactics to prevent and limit the spread of diseases and to promote production growth as well as yields (He *et al.*, 2020). Thus, antibiotics are widely used in animal husbandry, and various types of antibiotic resistance genes are frequently detected in livestock waste around the world (He *et al.*, 2020). The presence of antibiotics in the environment may provide long-term selective pressure for the emergence and transmission of these resistance-conferring bacteria (Colomer-Lluch *et al.*, 2011; He *et al.*, 2020). Though there was a notable numeric difference amongst the detected resistant genes in this study, there was no statistical significance observed among the classes of antibiotic resistant genes.

The *blaSHV* gene detection predominated the detection of *blaTEM* genes in samples from both Lebone and Sunflower hospice. The results conform with a study by Eftekhari *et al.*, (2012) on the detection of extended spectrum β -lactamase in urinary isolates of *Klebsiella pneumoniae*, in which SHV exceeded *blaTEM*. However, they are in disagreement with the findings by Yazdi *et al.*, (2012) who reported that the most prevalent β -lactamase-encoding gene was *blaTEM* followed by *blaSHV*. Thus, their increasing prevalence, as well as their shocking evolution seems to be directly linked to their clinical use (Pitout *et al.*, 2005; Ramos *et al.*, 2010). In addition, the gene *blaTEM* was also detected from *Pseudomonas aeruginosa* isolated from housefly samples (Hemmatinezhad *et al.*, 2015).

The antibiotic-resistant gene, *ermB* was the only detected macrolide resistant gene from housefly microbial communities captured in Lebone and Sparrow hospices. This gene is recognised as one of the four classes of *erm* resistance determinants that are correlated with disease causing microorganisms (Choi *et al.*, 2018). The detection of this resistant gene in housefly microbial communities signifies the presence resistant bacteria against macrolide. There is no surprise on this gene detection from housefly microbial communities because this gene has been detected in agricultural settings and primary animal production facilities (Choi *et al.*, 2018). Thus, housefly can easily be contaminated by antibiotic resistant bacteria due to their indiscriminatory movement.

The tetracycline resistance genes found at the highest frequency in gram-negative bacteria are related to efflux pumps, which are coded by the *tetA*, *tetB*, *tetC*, *tetD*, and *tetG* genes (Jahantigh *et al.* 2020). The occurrence of tetracycline resistance genes encoding ribosomal protection proteins was examined in housefly microbial communities from hospices in this study. Thus, the tetracycline efflux protein-encoding gene *tetA* was detected from all sampled hospices with Lebone hospice expressing high detection frequencies. In the study by Akter *et al.*, (2020), five antibiotic resistance genes were detected from three organisms isolated from houseflies, where *tetA* was the most common isolated resistant gene. Notably, the *tet* genes have been associated with anthropogenic impacts occurring in the surrounding, and a number of these genes have been found in various pollution sources. The presence of the *tetA* gene in all samples collected from all sampled hospices could be credited to its high abundance in the environment (Hubeny *et al.*, 2019). This efflux protein-encoding gene *tetA* has previously been shown to be present in both the hospitals and aquatic environments (Akter *et al.*, (2020).

Integrans play a major role in the rapid dissemination of multi-drug resistance amongst bacteria (Tansirichaiya *et al.*, 2016) and were investigated in this study. The results obtained in this study revealed the presence of class 1 and 2 integrans, as indicated by the presence of *Int1* and *Int2* gene from all sampled hospices. The high occurrence of integrans from hospices in this study may reflect that these classes 1 and 2 integrans are

widespread among housefly bacterial communities. Integrons have been found in approximately 9 % of the sequenced bacterial genomes. Class 1 Integron platform is the most ubiquitous and has been the most reported amongst clinical bacteria (Barlow *et al.*, 2004; Labbate *et al.*, 2009; Deng *et al.*, 2015). Although, class 2 integrons have been commonly reported in some species of Gram-negative organisms, they had significantly low occurrence and prevalence as compared with class 1 integron (Deng *et al.*, 2015). The occurrence of classes 1 and 2 integrons from the housefly bacterial communities was high in Lebone hospice, followed by Sunflower hospice and Sparrow hospice. The high occurrence of integrons in this hospice environment is of great concern because antibiotic-resistant determinants including integrons are known to be correlated with multidrug resistance bacteria (Huang *et al.*, 2020). The hospices house the immunocompromised and terminally ill patients who are very susceptible to infections caused by multidrug resistant pathogens.

Though, some resistant genes were detected in this study, there were no virulence genes detected for the selected bacterial pathogens in all three hospices investigated. Thus, according to published literature, there is no scientific consensus on the relationship between bacterial antibiotic resistance and virulence determinants (Higgins *et al.*, 2020; Beceiro *et al.*, 2013). There is no appropriate explanation of no detection of virulence genes in the current study samples. According to Higgins *et al.*, (2020), there is no clarity if the increase of antibiotic resistance does also increase the virulence determinants of pathogenic bacteria. However, both antibiotic resistance and virulence are necessary for bacterial survival under adverse conditions (Beceiro *et al.* 2013).

The obtained resistance gene sequences in this study appeared to be closely related with other resistance genes sequences found to be in various bacterial species and different cloning vectors. There was genetic variation observed from the detected genes when compared to those from GenBank (figures 6.7- 6.12) as well as from their position on the various tree topologies generated (figures 6.13 -6.18). This might be due to the horizontal gene exchange on mobile genetic elements including plasmids and transposons (Fukuda *et al.*, 2016). Thus, several studies suggested that the digestive tract of insects including

houseflies provides conditions conducive for horizontal transfer of antibiotic resistance genes on mobile genetic elements such as plasmids or transposons (Watanabe *et al.*, 1998; Hinnebusch *et al.*, 2002; Dillon and Dillon, 2004). Comparison of the obtained resistance genes sequences with those on NCBI, showed a high degree of similarity at the nucleotide level to several tetracycline, beta lactams, macrolides resistance genes and both the integrons. Analysis generated in this study has shown that resistant genes form well supported clades with corresponding congener. However, they somehow appear to be genetically distinct from other related resistant genes. More widespread analysis on the phylogeny of resistant genes is required because the microbial ecosystems are not isolated and there is extensive gene exchange between bacteria. It appears that more antibiotic resistance occurrences are most likely cases of acquired resistance, through the lateral transfer of antibiotic resistance genes from other ecologically and taxonomically distant bacteria.

In conclusion, houseflies are important vectors of several diseases (Manandhar and Gokhale, 2017). Thus, the detection of antibiotic resistant genes of bacterial pathogens from housefly samples collected from the hospices indicates the possible health risk to the hospice patients and the surrounding community at large. Therefore, it is imperative to assess the probable risk of dissemination of resistant genes in the environment including hospices. It is most important to understand that the transfer of resistant genes to other bacterial pathogens eventually invalidates the efficacy of current and upcoming antibiotics. Therefore, this leads to treatment failure for some life-threatening diseases caused by bacterial pathogens. Given the public health relevance, the results of this study reveal the importance and necessity of concerted surveillance of antimicrobial resistance and resistance genes in hospice environment around South Africa. It is therefore, recommended that necessary steps must be taken to control houseflies in these settings.

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CHAPTER 7

CONCLUSION AND RECOMMENDATIONS

The aim of this study was to conduct morphological and genetic characterization of houseflies (*Musca domestica*) found in hospices and to detect microbial pathogens of medical and veterinary importance that they are harbouring. Several objectives to attain this aim were identified. The results obtained in addressing the different objectives are summarily discussed below.

Objective 1: To conduct a systematic review and meta-analysis on bacterial pathogens identified from houseflies in different human and animal settings

The systematic review and meta-analysis study were conducted as a preliminary step to generate evidence and knowledge necessary to help formulate preventative measures against infectious bacteria harboured by houseflies. Published articles from 1980 to 2020 were retrieved from electronic databases and assessed for eligibility according to Preferred Reporting Items for Systemic Reviews and Meta-Analysis guidelines. Seventy-eight studies were included in the review with only 44 studies being eligible for meta-analysis. Results indicated that eligible studies used in this review were from four continents, i.e., Asia (47.4%) America (23.1%), Africa (20.5%) and Europe (8.9%). For meta-analysis, we focused on five pathogenic bacterial species including *Escherichia coli*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. We hypothesized that previous published studies from around the world report on vectoral potential of houseflies in the transmission of bacterial pathogens. The study has proved that indeed studies from around the world report on vectoral potential of houseflies in the transmission of bacterial pathogens. Thus, this provides more insight on the vectoral potential of houseflies in the transmission of medically important bacterial pathogens from different regions across the world.

Objective 2: To conduct morphological and genetic characterization of houseflies (*Musca domestica*) from different hospices by amplification of 16S rRNA, COI and ITS2 and subject them to phylogenetic analysis

This objective has provided a suitable framework for future studies on morphological and genetic characterization of houseflies found in hospices. Generally, there was high incidence of houseflies in the hospice with Lebone hospice having the highest occurrence as compared to Sparrow and Sunflower hospice. The high occurrence in Lebone hospice was mainly because of the agricultural activities including animal husbandry that occur near the hospice area. Therefore, there is a need of housefly control preferably by improving environmental sanitation and hygiene which can be done by elimination of housefly breeding sites and reduction of sources that attract houseflies from other areas around the hospices. In this study, *M. domestica* was identified morphologically and was further characterized genetically by the amplification of 16S rRNA, COI and ITS2 gene regions to supplement morphological identification. This confirmed the importance of considering both morphological and molecular identification of houseflies and other insects for taxonomic studies. The houseflies in this study are closely related to that of China, Korea and United Kingdom haplotypes. From the phylogenetic tree constructed, the isolates from this current study showed a close relationship with *M. domestica* strains from various countries including China, Korea and United Kingdom. Furthermore, genetic distance analysis showed no differences in between houseflies from three sampled hospices. The eligibility of the 16S rRNA, COI and ITS2 regions was assessed as a marker for species identification for houseflies collected from hospices in Bloemfontein and Johannesburg, South Africa. The results showed that 16S rRNA, COI and ITS2 were able to sufficiently determine or identify *M. domestica* to species level in this study. However, the 16S rRNA and ITS2 seemed to have higher levels of interspecific diversity than COI. The current study also suggests the need to supplement COI data especially in closely related species. We hypothesized that there is a variation in the abundance of houseflies from the sampled hospices in the different provinces due to differences in climate and vegetation. However, there was no significant variation in the abundance of houseflies observed from the sampled hospices due to difference in climate and vegetation in the different provinces. Thus, more analysis on the phylogeny of houseflies and the population dynamic studies are also recommended. Future studies should

consider the characterization of houseflies from hospices in the other cities of South Africa to determine the relative abundance of these flies in different geographic locations.

Objective 3: To compare the gut microbiota of houseflies (*Musca domestica*) collected from different hospices by 16S *rRNA* metagenomic analysis using next-generation sequencing

The characterization of the bacterial communities associated with housefly gut using partial 16S *rRNA* gene amplicon sequencing was conducted. The most abundant phyla detected with variable abundance observed among all houseflies were Proteobacteria, followed by Firmicutes and Bacteroidetes. The class Alphaproteobacteria was observed from fewer samples from Sparrow and Sunflower hospices as compared to Lebone hospice. The present study showed the diverse composition of bacterial communities in the gut of houseflies obtained in hospices within the two large cities namely, Bloemfontein and Johannesburg. Some genera are pathogenic to humans and animals. We hypothesized that houseflies (Muscidae) from hospices in South Africa are linked to a variety of bacterial communities. The hypothesis was proved to be correct due to various bacterial genera detected in houseflies from all hospices in South Africa. Thus, the presence of houseflies in hospices surroundings present a health hazard of pathogenic bacteria to patients housed within the institution and the community at large. Therefore, it is of importance to make an effort to limit housefly breeding by employing proper sanitation. Furthermore, fly traps particularly sticky traps should be used to control the flies during warmer seasons as they are cost efficient and easy to setup.

Objective 4: To detect antibiotic resistance and virulence genes from housefly (*Musca domestica*) DNA by species-specific PCR assays

In this section, the occurrence of antibiotic resistance and virulence genes in housefly microbial communities from three hospices was determined using PCR assays. The detected resistant genes and integrons (*blaTEM*, *blaSHV*, *tetA*, *ermB*, *intl* and *intlI*) were subjected to sequencing. Gene sequences were edited and subsequently subjected to BLASTn to confirm their identities. Though, some resistant genes were detected in this study, there were no virulence genes detected for the selected bacterial pathogens in all

three hospices investigated. Scientific literature highlight that, there is no scientific consensus on the relationship between bacterial antibiotic resistance and virulence determinants. Thus, there is no appropriate explanation of no detection of virulence genes in the current study samples. The successful detection of bacterial antibiotic-resistant determinants by PCR assay from housefly DNA in this study is of great concern. Antibiotic-resistant determinants including integrons are known to be correlated with multidrug resistance bacteria. Findings from the present study indicate that houseflies from hospices have potential to disseminate antimicrobial-resistant pathogens to humans housed within the institution. We hypothesized that houseflies are potential mechanical or biological vectors for the dissemination of virulent and antibiotic-resistant bacteria. The presence of antibiotic resistant genes detected in this study confirm the potential of houseflies to act as vector to disseminate virulent and antibiotic-resistant bacteria. Thus, the hypothesis was proven to be correct. Therefore, this study recommends the prudent use of antibiotics which may lead to a decrease in the prevalence of antibiotic resistance genes in bacterial populations.

Recommendations

This thesis has provided a suitable framework; therefore, we recommend the following future and possible studies addressed below.

- To investigate different housefly control techniques inside and outside hospice buildings.
- To culture and isolate the bacterial species of medical importance from hospice houseflies to enable genotyping of these bacterial species.
- To conduct phenotype antibacterial resistance studies from cultures to compare with genetic analysis for accurately identifying antibiotics from which the bacteria has developed resistance.

Bacterial pathogens identified from houseflies in different human and animal settings: A systematic review and meta-analysis

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Abstract

Housefly (*Musca domestica*) is an excellent candidate for the distribution of susceptible and resistant bacterial strains that potentially threaten public health. To date, there is a paucity of information on the global distribution of pathogenic bacteria of medical and veterinary importance from diverse environmental settings. Therefore, this study was undertaken to conduct a systemic review and meta-analysis to estimate occurrence of various bacterial species of medical and veterinary importance harboured by houseflies around the world. Published articles from 1980 to 2020 were retrieved from electronic databases and assessed for eligibility according to Preferred Reporting Items for Systemic Reviews and Meta-Analysis guidelines. Seventy-eight studies were included in the review with only 44 studies being eligible for meta-analysis. Results indicated that eligible studies used in this review were from four continents, i.e., Asia (47.4%), America (23.1%), Africa (20.5%) and Europe (8.9%). The majority of the studies (56.4%) used the culture method for the identification of bacterial pathogens, while 30.7% used both culture and PCR techniques. For meta-analysis, we focused on five pathogenic bacterial species including *Escherichia coli*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*. High heterogeneity was found among studies investigating different pathogens including *E. coli* ($Q = 10,739.55$; $I^2 = 99.60$; $Q-p < 0.0001$), *E. faecium* ($Q = 317.61$; $I^2 = 86.46$; $Q-p < 0.0001$), *K. pneumoniae* ($Q = 1,576.61$; $I^2 = 97.27$; $Q-p < 0.0001$), *S. aureus* ($Q = 2,439.12$; $I^2 = 98.24$; $Q-p < 0.0001$) and *P. aeruginosa* ($Q = 1,283.0$; $I^2 = 96.65$; $Q-p < 0.0001$). Furthermore, it was observed that houseflies carried a considerable number of susceptible and antibiotic-resistant bacterial strains that pose considerable threats to public health. Findings from this study have provided more insight on the vectoral potential of houseflies in the transmission of significant bacterial pathogens from different regions across the world. Further investigation is required to quantify the bacterial contamination and dissemination by houseflies.

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APPENDIX 2



Morphological features of *Lucillia cuprina* captured from hospices.



Morphological features of the *Lucillia sericata* captured from hospices