Chapter 1

LITERATURE REVIEW, RESEARCH AIMS AND OBJECTIVES
1.1 THE HOUSE FLY (*Musca domestica*)

1.1.1 Description and life cycle: Biology, Habitat and Behavior

House flies are one of the most abundant and important groups of Diptera insects of the family *Muscidae* (Skidmore, 1985; Moon, 2002). House fly (also housefly, house-fly or common housefly), originated on the steppes of central Asia, but now occurs on all inhabited continents, in all climates from tropical to temperate, and in a variety of environments from rural to urban (Capinera, 2008). They have a complete metamorphosis with distinct egg, larva or maggot, pupa and adult stages. Each female fly can lay approximately 500 eggs in five to six batches of about 75 to 150 (Service, 2000) in warm, moist material that will supply appropriate food for the larval development. The Eggs are white and about 1.2 mm in length; usually hatch in 12-24 hours. Larvae (maggots) hatched from the eggs, live and feed on organic material (dead and decaying), such as garbage or feces. They are pale-whitish, 3–9 mm long, thinner at the mouth end, and have no legs. They live at least one week. Larvae possess porous pharyngeal ridges in their cephalopharyngeal skeleton that is used to filter essential food particles and bacteria from the liquid substrates (Moon, 2002). House flies reproduce and develop as larvae in decaying organic matter such as animal manure, human refuse, open privies, soiled animal bedding, litter and waste around food and vegetable processing plants, which all are areas teeming with diverse and active microbial communities (Greenberg, 1973; Graczyk et al., 2001; Moon, 2002). All developmental stages of house flies (e.g. larvae, pupae, adults) are commonly contaminated with various microorganisms. House flies require an active microbial community for larval development (Schmidtmann & Martin, 1992; Zurek et al., 2000). At the end of their 3rd instar, the maggots crawl to a dry cool place and transform into ≈8 mm long pupae, colored reddish or brown. During the pupation process, the 3rd larval integument becomes hard and forms the puparium (Frankel & Bhaskaran, 1973; Moon, 2002). The adult flies emerge from the pupae and live from two weeks to a month (Kettle, 1995). The adults are usually gray, less than 1/4-inch long with four black stripes on the thorax and whole body is covered with hair-like projections (Fig. 1.1). The adult house fly, *Musca domestica*, is a very common pest of great economic importance (Farkas et
and a non-biting insect found in close association with humans throughout the world. Houseflies feed on liquid, semi-liquid substances (rich in carbohydrates), solid material (feces, open sores, sputum), and moist decaying (spoiled food, eggs, fruit, and flesh) organic matter (Dübendorfer et al., 2002). They spit out saliva or vomit on solid foods in order to soften or to predigest it. They also regurgitate partly digested matter and pass it again to the abdomen (Moon, 2002).

![Figure 1.1 The housefly (Musca domestica)](image)

**Figure 1.1 The housefly (Musca domestica)**

(Courtesy - The Natural History Museum, London)

### 1.1.2 House fly as a transmitter of disease

House flies (*Musca domestica* L.) are cosmopolitan, ubiquitous, synanthropic insects that serve as mechanical or biological vectors for various microbes. House flies (*Musca domestica* L.) reproduce and develop in decomposing organic waste and therefore is constantly in contact with different species of microorganisms. These flies have long been considered vectors or transporters of pathogenic microorganisms (West, 1951), due to their coprophagous habits. These flies live in close proximity to human and animal habitats; they pose a risk of transmitting potential pathogenic microorganisms from diseased sources to new environments. They have been implicated in the transmission of serious diseases such as anthrax, ophthalmia, typhoid fever, tuberculosis, cholera, and infantile diarrhea (Scott & Lettig, 1962; Greenberg, 1965; Keiding, 1986; Graczyk et al., 2001) and have been demonstrated to harbor or transmit other pathogenic bacteria including,
Salmonella spp. (Greenberg, 1971; Bidawid & Edesen, 1978; Mian et al., 2002), Proteus spp., Shigella spp. (Greenberg, 1971; Bidawid & Edesen, 1978, Levine & Levine 1991), Chlamydia spp., Campylobacter jejuni (Shane et al., 1985), Klebsiella sp. (Fotedar et al., 1992; Sulaiman et al., 2000), Escherichia coli O157:H7 (Grubel et al. 1997; Buma et al. 1999; Kobayashi et al. 1999; Sasaki et al. 2000; Alam & Zurek, 2004; Szalanski et al. 2004; Ahmad et al., 2007), Vibrio cholerae (Escheverria et al., 1983; Fotedar, 2001), Campylobacter fetus (Rosef & Kapperud, 1983), Aeromonas caviae (Nayduch et al., 2001), Yersinia pseudotuberculosis (Zurek et al., 2001), enterococci (Macovei & Zurek, 2006; Macovei et al., 2008; Graham et al., 2009) and Helicobacter pylori, a causative agent of gastric ulcer (Li & Stutzenberger, 2000). Recently, these insects were reported to be involved in disease outbreaks including E. coli O157:H7 in Japan (Sasaki et al., 2000) and Vibrio cholerae in India (Fotedar et al., 2001). They are also implicated in the transmission of protozoan infections such as amebic dysentery (Szostakowska et al., 2004); helminthic infections such as pinworms, roundworms, hookworms and tapeworms, as well as viral and rickettsial infections (Greenberg, 1971; Gregorio, 1972; Greenberg, 1973; Graczyk et al., 2001). Moreover, house flies have been reported as mechanical vector of nosocomial infections of multidrug-resistant bacteria in clinical settings (Fotedar et al., 1992; Rady et al., 1992). Since adult house flies are highly mobile (disperse up to 125 km) (Winpisinger et al., 2005; Chakrabarti et al., 2010), they transport microbes from septic environments to other substrates via contamination of their surfaces (feet, wings, bodies) and also by regurgitation of crop contents (Moon, 2002). Their persistent and ubiquitous association with humans, animals, food, refuse and excreta make flies potential mechanical or biological vectors for the dissemination of pathogenic and multidrug-resistant bacteria (Zurek et al., 2000; Graczyk et al., 2001; Alam & Zurek, 2004; Rahuma et al., 2005; Macovei & Zurek, 2006; Macovei et al. 2008; Chakrabarti et al. 2010; Ahmad et al. 2011).

1.1.3 House fly gut microbiology

The guts of many insects are characterized by a high diversity of microflora (Bignell, 1984). In many instances, the association between microorganisms and
insects is casual and transitory, where microorganisms are probably derived from
the diet upon which the insect feeds. In other cases, the relationship between
certain microorganisms and their insect hosts is more intimate and specific. Many
insects, across a broad taxonomic range, possess obligate microbial
endosymbionts that benefit the insect in some way (Douglas, 1989). House flies
feed on septic substrates; bacteria can be harbored internally and later transmitted
via regurgitation and/or defecation. The alimentary canal of the flies includes a
highly modified crop that branches from the stomadaeum and extends to the
abdomen. The crop is a bivalved sac believed to function primarily for storage of
sugars utilized for flight (Singh & Judd, 1965). The crop of the house fly has been
observed as an important site of bacterial accumulation (Kobayashi et al., 1999;
Sasaki et al., 2000; McGaughey & Nayduch, 2009). Further, the crop is important
in *M. domestica* because of the fly’s method of feeding. Because the fly
regurgitates when feeding, any bacteria present in the crop are readily deposited
on the flies’ food source (Graczyk et al., 2001). Flies also frequently defecate on
food sources and microbes that have survived to the rectum are passed in this way
as well (Kobayashi et al., 1999; Sasaki et al., 2000; Graczyk et al., 2001). Because
of their high intake of food, they deposit feces constantly, one of the factors that
make the insect a dangerous carrier of pathogens. The association of living
bacteria within the alimentary canal or/and on the body surface of house flies, and
their transmission, has been demonstrated through various studies. Some of the
microbes that house flies carry within their bodies are pathogenic (Greenberg et
al., 1970). Historically, the first detailed observations of this nature were made by
Graham-Smith (1910) who caught flies randomly, artificially infected them with
pathogenic bacteria, and recorded their recovery over time. Early studies by
McGuire & Durant (1957) found approximately 20 times more internal than
external bacteria in *M. domestica*, denoting on the role of spatial location of
microbes in their survival. There have been several qualitative reports of microbial
communities isolated from house fly surveys (Sulaiman et al., 2000; Nazmi et
al.,2005; Rahuma et al., 2005; Vazirianzadeh et al., 2008; Butler et al., 2010).
Recent studies (Kobayashi et al. 1999; Nayduch et al. 2002) have focused on
tracking the fate and proliferation of selected bacteria in the fly gut. Kobayashi et
al., (1999) observed evidence of the *E. coli* O157:H7, actively proliferating on the labellum and in the crop. Nayduch *et al.* (2002) tracked the persistence and transmission of *Aeromonas caviae* and observed the viable bacteria in the crop as well as the midgut (McGaughey & Nayduch, 2009). These studies showed the persistence and potential role of house flies as vectors of potential pathogenic bacteria in the gut of flies. It was observed that the bacteria survive transiently in the fly alimentary canal, actively proliferate and are frequently deposited in vomitus and in some cases in excreta as well (Kobayashi, 1999; Sasaki, 2000). Survival, persistence, proliferation and passage of bacteria from the fly gut directly impacts biological vector potential.

### 1.2 THE FLESH FLY (*Sarcophaga* sp.)

#### 1.2.1 Description and life cycle: Biology, Habitat, and Behavior

Flesh flies (*Sarcophaga* sp.) belong to the order Diptera, family Sarcophagidae (Pape, 1996). The flies belonging to the genus *Sarcophaga* are common filth flies with a cosmopolitana distribution. They breed in carrion, dung, or decaying material, but a few species lay their eggs in the open wounds of mammals (Pape, 1996), hence their common name. They have holometamorphic life cycle and are larviparous. Flesh-flies, being viviparous, frequently give birth to live young on corpses of human and other animals, at any stage of decomposition from recently dead through to bloated or decaying. Larvae of flesh flies are the scavengers and are also called as maggots. The adult flesh flies can be recognized with naked eyes due to their larger body size with the longitudinal stripes on thoracic region. The main character for confirmation is the presence of line of hypopleuron bristles on lateral side of thorax (Fig. 1.2). These flies are generally found near unhygienic sites such as flesh markets, slaughter houses, slum areas, garbage, kitchen refuses, faecal matter, cow dung or cattle droppings, sewage treatment plants, decaying coconut and sweets (Joseph & Parui, 1980).

The duration of the different stages (larva, pupa and adult) of life cycle vary from species to species and according to environmental conditions such as temperature, humidity soil texture and choice of flesh to deposit larvae. There are food
preferences within species (Joseph & Parui, 1980). Flesh fly maggots occasionally eat other larvae although this is usually because the other larvae are smaller and get in the way. Flesh flies and their larvae (Fig. 1.2) are also known to eat decaying vegetable matter and excrement and they may be found around compost piles and pit latrines (Pape, 1996).

For identification purpose, generally, male specimens are chosen and the main character used is male genitalia. The use of only morphological characters makes it difficult for proper and correct identification of the species. Recently, the identification has been possible with DNA sequencing, although this is at a preliminary stage with many species yet to be study (McIntosh, 2007). Comparatively, no prominent and distinct morphological characters are seen in flesh flies. The bristle counts generally are taken as key characters along with the size of organs, coloration on antennae, thorax and the coloration patterns on abdomen to identify the genera and species of these flies. The Indian Sarcophagid fauna is so far known to be comprised by 163 species under 50 genera out of which 21 species have been reported from Maharashtra, India (Nandi, 2002).

Figure 1.2 The fleshfly (Sarcophaga sp.) [Larval & Adult stage]
(Courtesy - Ohio State University, USA)
1.2.2 Brief description of body parts-identification

Head is broad with mobile neck. It mainly contains compound eyes (Chapman, 1999), antennae (Joseph & Parui, 1980), and mouth parts. Compound eyes are very large brown or red in colour, bear uniform ommatidia (Chapman, 1999). Antennae are sensory organs, placed in a round antennal socket. At the base of lunule there is a pair of antennae having three segments on each antenna (Joseph & Parui, 1980). Arista commonly plumose on basal half, bare in a few species. Microscopic view shows hairy arista, which becomes narrow at tapering end. It is a part of sensory organ (Khole, 1975). Palpi are an appendage attached to an oral part and serving as an organ of sense (Khole, 1973). Mouth parts are typically sponging and sucking type and represented by proboscis. This is flexible and work as suction pump (Joshi & Ranade, 1973). In male-female thorax is of grey colour containing distinct black stripes. There are the three segments horizontally, which divides thorax in Prothorax, Mesothorax, and Metathorax. Sarcophagids mainly contain hypopleuron (a continuous line of bristle). Three pairs of legs are present. Hind legs are longer as compared to fore legs and middle legs. Male has longer legs than female and contains disc at the tips, which plays important role during mating period. All legs are clothed with thick short bristles. A pair of wings is present. They are present on Mesothorax with a texture that is smooth hyaline, transparent with slight yellowish tinge. Cells are formed due to venation on wings structure and size of cells changes as per the species. Halteres are also known as balancers. Halteres are of dumbbell shape and off-white sometimes yellow-brown in colour. It is a gyroscopic organ and controls the flight of the fly. Hypoplueral bristles- a line of bristle constitutes the most important character for the identification of family Sarcophagidae.

Differentiation of male and female specimens depends on abdomen and can be made with the naked eyes. Abdomen is black or grey in color with checkered marbled pattern of silver colour sometimes golden colour. Male has longer abdomen tapering at the end and curved towards ventral side its second genital segment is of black or of orange/red colour. This is a very important character for differentiating male specimens. In females, abdomen is broad and rounded, short.
in length as compared to males. Genitalia (Adaeagus or penis) - Taxonomically in males, genitalia is an important character. It contains two genital segments (G1, G2), inner-outer forceps, penis, anterior-posterior paramere and marginal bristles. Genitalia are always present inside the abdomen and just the 1st genital segment can be seen outside but at the time of mating, it exerts out. Penis contains paraphallus, styli of glans, theca of penis, and ventralia. The small auditory organ is located on the ventral prosternum. From the sense organs, the sensory information is transmitted to the brain via ascending interneurons. Anatomical organs like crop, which is a non-respiratory organ functions as an air sac and also supplies more oxygen and performs thermoregulation (Khole, 1973). The flies respire through trachea (respiratory organ). Comparatively, Sarcophagidae has more number of tracheas than other insects as they are larviparous, and the number ranges from 8-10 (Khole, 1975). In reproductive biology, female flesh flies show stages of oogenesis where the ovary of *Sarcophaga ruficornis* possesses about 20-30 ovarioles on each side (Verma, 1984).

### 1.2.3 The Flesh fly- medical, economic and forensic importance

Flesh flies have great importance from a hygienic point of view. They are necrophagous insects well known for their role in myiasis and are considered as carrion flies of forensic importance as they offer clues for the estimation of the postmortem interval (PMI). Sarcophagidae (flesh flies) have been incriminated as a public health concern in many parts of the world (Sukontason et al., 2006). Due to their life cycle of synanthropic behaviour and their feeding habit which is closely associated with the human environment, adult flies in these groups could be mechanical carriers of numerous pathogenic microorganisms from filth to human food, thereby causing diseases in humans (e.g., Greenberg 1971; Levine & Levine 1991; Sukontason et al. 2000; Sulaiman et al., 2000). They are the vectors of many diseases and cause dangerous diseases like myiasis in animals, mostly in sheep, horses, dogs, cattle, humans and also give them blood poisoning, or asymptomatic leprosy infections (Hall & Wall, 1995). Other diseases that have been reported to be transmitted by them include typhoid, paratyphoid, cholera, amoebic giardial and balantidial dysentery, taeniasis, ascariasis, enterobiasis,
enteric fevers (Joseph & Parui, 1980). The flies have been reported to attack invertebrates like grasshopper and snails. They are the potential vectors of rabbit hemorrhagic disease virus (RHDV) in New Zealand (Henning et al., 2005). Surprisingly, some people in the West consume these maggots as food. Flesh flies can carry leprosy bacilli and can transmit intestinal pseudomyiasis to people who eat the flesh-fly larvae (Hall & Wall, 1995).

Economically also, flesh flies are very important. In western countries, maggot therapies are still given to remove necrotic tissue from wound. Maggot therapy is also known as maggot debridement therapy (MDT) which involves the intentional introduction of live, disinfected maggots into the non-healing skin and soft tissue wound. They create proteolytic enzymes that liquefy necrotic tissue, and absorb the resulting semi-liquid material within a few days. Some enzymes, which are used in production of medicine such as polypeptide, are obtained through cultivation of an insect cell line established from flesh fly embryo. Anglers use maggots as bait to catch non-predatory fish in Europe (Wyatt & Hall, 2009). The use of maggots and diatoms conjointly has proved to be an important factor in the medico legal cases referred with advance decomposition (Singh & Kulshreshtha, 2004). The presence of these flies is the best indication of unhygienic condition. Among all necrophagous insects, flesh flies (Diptera: Sarcophagidae) have special forensic importance. Immature stages of sarcophagids are often encountered on dead bodies and they are important forensic indicators to determine the post-mortem interval (PMI). The larvae of sarcophagids showing association with the carrion can play significant forensic role if their age is determined accurately (Byrd & Castner, 2001). Flesh flies larvae, commonly known as maggots, live for about 5–10 days, before descending into the soil and maturing into adulthood. At that stage, they live for 5–7 days. The life cycle of flesh-fly larvae has been well researched and is very predictable. Different species prefer bodies in different states of decomposition, and the specific preferences and predictable life cycle timings allows forensic entomologists to understand the progress of decomposition and enables the calculation of the time of death by back extrapolation. This is done by determining the oldest larva of each species present, measuring the ambient
temperature and from these values, calculating the earliest possible date and time for deposition of larvae. This yields an approximate time and date of death (D.O.D.). This evidence can be used in forensic entomology investigations and may assist in identification of a corpse by matching the calculated time of death with reports of missing persons. Such evidence has also been used to help identify murderers (Pape, 1996).

1.3 POLYPHASIC APPROACH

1.3.1 Characterization of bacterial strain for taxonomic purpose

Taxonomy relies on three key elements: characterization, classification and nomenclature. The characterization of a strain is a key element in prokaryote systematic (Tindall et al., 2010). A strain or set of strains shown to be novel taxa should be comprehensively and properly characterized. The goal of this complete characterization is to place them within the hierarchical framework laid down by the Bacteriological Code (1990 revision) (Lapage et al., 1992), as well as to provide a description of the taxa.

The term “polyphasic” coined by Colwell in 1970, refers to the integration of genotypic, chemotaxic and phenotypic information of a microbe in order to perform reliable grouping of the organism. A polyphasic approach is used for bacterial systematic and complete characterization in order to provide a description of the new taxa. This approach is presently the most accepted for determining taxonomic position of bacteria and several microbes. Genotypic, chemotaxonomic and phenotypic tests for bacterial systematics include aspects such as complete 16S rRNA gene sequencing and its comparative analysis by phylogenetic trees, DNA-DNA hybridization studies with related organisms, analyses of molecular markers (protein-encoding genes) and molecular fingerprinting, biochemical assays, physiological and morphological tests as well as methods of chemical analysis of the cell.
1.3.2 Genetic based characterization

Modern bacterial taxonomy has been strongly influenced by developments in genetic methods. Several DNA-based typing methods are known; these provide information for delineating bacteria into different genera and species and have the potential to resolve differences among the strains of a species. Therefore, newly isolated strains must be classified on the basis of the polyphasic approach. The development of nucleic acid hybridization methods (DNA–DNA & DNA–RNA) has allowed the indirect comparison of gene sequences. The introduction of the analysis of the 16S rRNA gene by cataloguing (Fox et al., 1977), reverse transcriptase-sequencing (Sanger et al., 1977; Lane et al., 1988) and finally PCR-based gene sequencing (Saiki et al., 1988) has provided a useful working hypothesis on which other features (phenotypic and chemotaxonomic) may be compared when investigating the taxonomy and evolution of prokaryotes. It is realistic to assume that the recognition of novel taxa often centres on the use of 16S rRNA gene based techniques. Despite the widespread use of 16S rRNA gene sequencing, there are a number of points that need to be considered when evaluating the data. 16S rRNA gene sequences are one of the most widely used datasets. Although there is justification for using 23S rRNA gene sequences, this dataset is currently much smaller and the 16S rRNA gene sequence presently remains the gene sequence of choice. As more whole genome sequences become available, a greater selection of genes with different degrees of resolution will become available (Tindall et al., 2010).

There is extensive documented evidence that two strains sharing less than 97 % 16S rRNA gene sequence similarity are not members of the same species (Amann et al., 1992; Collins et al., 1991; Fox et al., 1992; Martínez-Murcia & Collins, 1990; Martínez-Murcia et al., 1992). 16S rRNA gene sequences alone do not describe a species, but may provide the first indication that a novel species has been isolated (less than 97 % gene sequence similarity). Where 16S rRNA gene sequence similarity values are more than 97 % (over full pairwise comparisons), other methods such as DNA–DNA hybridization or analysis of gene sequences with a greater resolution (protein-encoding genes) must be used. These methods
must also be correlated with the characterization based on phenotypic and chemotaxonomic tests (Tindall et al., 2010). The resolving power of the 16S rRNA gene with respect to the delineation of novel species has been extensively debated. As indicated above, there is growing interest in the use of other genes with a greater degree of resolution (protein-encoding genes) to resolve issues that are not solved by 16S rRNA gene sequencing. Sequences of other conserved protein-encoding genes, typically housekeeping genes, can provide a higher resolution than 16S rRNA gene sequences and can complement DNA–DNA relatedness or 16S rRNA gene sequence data for taxonomic analysis at the species level. The technique of DNA–DNA or DNA–RNA hybridization was introduced into prokaryote systematics from the 1960s onwards (Brenner et al., 1969; De Ley et al., 1966; Johnson & Ordal, 1968; McCarthy & Bolton, 1963; Pace & Campbell, 1971; Palleroni et al., 1973). DNA–DNA hybridization (DDH) is to be performed in cases where the new taxon contains more than a single strain, in order to show that all members of the taxon have a high degree of hybridization among each other. DDH is necessary when strains share more than 97 % 16S rRNA gene sequence similarity. If the new taxon shows this high degree of similarity to more than one species, DDH should be performed with all relevant type strains to ensure that there is sufficient dissimilarity to support the classification of the strain(s) as a new taxon (Grimont et al., 1980; Rosselló-Móra, 2006). A DDH value equal to or higher than 70 % has been recommended as a suitable threshold for the definition of members of a species (Brenner, 1973; Johnson, 1973; 1984; Wayne et al., 1987), but this value should not be used as a strict species boundary (Ursing et al., 1995). A single species must embrace all the strains that cannot be clearly discriminated by a stable phenotypic property. A single species may embrace groups of strains with DNA–DNA hybridization values of less than 50 % that are indistinguishable by means of other properties tested at the time. A single species may contain several genomic groups or genomovars (Ursing et al., 1995) that may be reclassified as novel species once clear and stable discriminative phenotypic properties are found. DNA G+C content is still a useful parameter and is one of the classical genotyping methods in the bacterial systematics. It is also an important prerequisite for determining the
conditions used in DNA–DNA hybridizations. The variation in the percent GC content is not more than 3 % within a well-defined species and not more than 10 % within a well-defined genus and it varies from 24 to 76 % in the bacterial world (Wayne et al., 1987).

Advances in DNA-based molecular techniques have revolutionized the bacterial identification and field of bacterial taxonomy. Nucleic acid fingerprinting methods provide information at the subspecies and/or strain level. Examples for these techniques are: amplified fragment-length polymorphism PCR (AFLP), macrorestriction analysis after pulsed field gel electrophoresis (PFGE), random amplified polymorphic DNA (RAPD) analysis, rep-PCR (repetitive element primed PCR, directed to naturally occurring, highly conserved, repetitive DNA sequences, present in multiple copies in the genomes) including REP-PCR (repetitive extragenic palindromic-PCR), ERIC-PCR (enterobacterial repetitive intergenic consensus sequences-PCR), BOX-PCR (derived from the boxA element), (GTG)5-PCR and ribotyping. A major disadvantage of some of these fingerprint-based methods is that the results are often very difficult to compare when they have been obtained in different laboratories due to the lack of standardization. DNA fingerprinting methods are of limited value for species descriptions, but when used properly they can be valuable for identification at the species and subspecies levels. These typing techniques can however be very useful to demonstrate whether or not isolates of a novel taxon are members of a clone.

1.3.2 Phenotypic characterization

A. Morphology, physiology and biochemistry

Examinations of morphological, physiological and biochemical properties are the oldest tools for the characterization and classification of prokaryotes. All relevant traits should be listed in the protologue of each taxon being described. At the rank of species and subspecies, more than one representative strain should be included in order to determine which characteristics are stable and which are variable. Morphological, physiological and biochemical traits should be carefully evaluated to determine those that are common (or even unique) to the taxon in question.
Properties that are variable may be included in the protologue since they may indicate subgroupings within the taxon in question.

**B. Analysis of morphological traits**

These methods utilize colony and cell morphology to obtain an initial identification of a microorganism. This is accomplished through simple isolation and culturing of the microorganism and subsequent visual observation using microscopy. The morphological properties include: shape, size, surface characteristics and pigmentation, cell wall characteristics (Gram-staining), sporulation characteristics, mechanisms of motility and, other cellular inclusions and ultrastructural characteristics.

The diversity in cell form and its underlying structural basis are now becoming apparent, largely due to a combination of improved methods of visualizing prokaryote cells, structural studies at the subcellular and molecular levels, and genome sequencing. Although light microscopy may be adequate for describing coarse features of the cell, electron microscopic images have a higher resolution. Depending on the organisms concerned, scanning electron micrographs may be used to show the morphology of whole cells, while transmission electron micrographs may be used to determine the infrastructure of the cell envelope or the presence of cytoplasmic inclusions, internal membrane structures, etc. Cell shape and form should be adequately described and supported by appropriate photographs (including electron micrographs). Special attention should be given to characteristic features of the cell, such as apolar growth, the presence of stalks, or the fact that they undergo a life cycle. The formation of prosthecae, budding or branching are also important characteristics, as is the formation of cell aggregates (e.g. diplococci, sarcinas, chains of cells) and formation of spores-endospores or exospores. In the case of endospore-forming organisms, the location of the spores and their size in relation to the size of the vegetative cell should be noted. In the case of exospores, features such as the formation of sporangia and the shape of the spores should be noted. The location of flagella should be noted with care: polar, subpolar or laterally inserted. Their occurrence in one location (tufts of flagella) or
distributed at different locations should be noted. Special care should be taken to check whether strains are motile by standard tests or under the light microscope before the strain is examined in an electron microscope since flagella may be lost during fixation. Motility should be described accurately since both the form and speed may be of significance. Some organisms may be rapidly motile, others only slowly. A distinction should be made between motility caused by flagella and gliding or other forms of motility. Intracellular structures visible under the light microscope such as gas vacuoles, sulphur granules or polyhydroxybutyrate granules should be investigated using appropriate methods. When grown on solid surfaces, the shape and size of the colony, together with other features of the colony form, should be accurately noted. Standard textbooks cover the diversity of colony shape and size, including features such as the nature of the outer edge, whether the colony is opaque or translucent and whether the colony has any visible additional structures/features. Some strains form rapidly spreading colonies, while others are fairly compact. In cases where colonies are not formed, it is still important to record whether cell material is pigmented. Cellular pigments may be soluble either in water or organic solvents. Typical pigments include carotenoids (which turn blue in the presence of concentrated sulphuric acid), flexirubins (which change colour reversibly under acid and alkaline conditions), (bacterio) chlorophyll [which is soluble in organic solvents, but becomes water soluble following saponification; treatment with acid results in the formation of (bacterio) phaeophytin], melanin and pyocyanin (fluorescence at 360 nm, reversible colour change at acid/alkaline pH); this list is not exhaustive. The Gram stain (Gram, 1884) is one of the oldest forms of staining the cells of prokaryotes and distinguishes between cell-wall structures that allow a dye complex to be washed out of the cell and those that retain it.

C. Analysis of physiological and biochemical characteristics
Phenotypic identification methods include the study of the physiological and biochemical properties of a microorganism by testing its growth requirements and enzymatic activities. The biochemical tests use specific growth media, nutrients, chemicals or growth conditions to elicit an observable or measurable biochemical
response from the microorganism, thereby enabling its identification and
coloration. These tests include: utilization of carbon and nitrogen sources,
growth requirements (anaerobic or aerobic; temperature-optimum and range, pH
optimum and range), preferred osmotic conditions, generation of fermentation
products, production of enzymes, production of antimicrobial compounds, as well
as sensitivity to metabolic inhibitors and antibiotics. Examples of recognized tests
include: phenol red carbohydrate, catalase and oxidase tests, oxidation-
fermentation tests, methyl red tests, Voges-Proskauer tests, nitrate reduction,
starch hydrolysis, tryptophan hydrolysis, hydrogen sulfide production, citrate
utilization, litmus milk reactions, etc. Several miniaturized and automated
commercial systems such as API (bioMérieux) tests or Biolog (Biolog Inc.) test
plates are currently available with well-defined quality control procedures that
allow for rapid identification of microorganisms. Physiological and biochemical
tests should be carried out in test media and under conditions that are identical or
at least comparable. The expression of microbial phenotypes is highly dependent
on environmental variables (e.g., culture pH, temperature, selective vs non-
selective media, depletion of nutrients, presence of stressors etc.), and thus, may
introduce inconsistencies in the identification process. The phenotypic methods
are only acceptable if the response criteria are sufficient to identify the
microorganism with a high level of confidence and distinguish it from
phylogenetically close relatives that potentially pose safety concerns. Also, the
applicability of the method is based on the robustness of information in reference
databases. As such, results from phenotypic methods may require supporting data
from other methods to accurately identify a microorganism.

1.3.3 Chemical characterization
The chemical composition of the cell (fatty acid, polar lipid and respiratory
lipoquinone composition, amino acid composition of the peptidoglycan of the cell
wall of Gram-positive bacteria, presence and size of mycolic acids, polyamine
pattern, etc.) is included under a separate heading, chemotaxonomy, but it is in
essence a part of the phenotypic characterization of an organism. Chemical
characterization of the cell (traditionally referred to as chemotaxonomy) deals with
various structural elements of the cell including the outer cell layers (peptidoglycan, techoic acids, mycolic acids, etc.), the cell membrane(s) (fatty acids, polar lipids, respiratory lipoquinones, pigments, etc.) or constituents of the cytoplasm (polyamines).

A. Analysis of Fatty Acid Methyl Ester composition (FAME analysis)
Microorganisms can be identified by analyzing the fatty acid profiles of whole cells or cell membranes using gas-liquid chromatography or mass spectrometry. The data on the type, content, proportion and variation in the fatty acid profile are used to identify and characterize the genus and species by comparing it against the fatty acid profiles of known organisms. Fatty acids, ester-linked to the glycerol are typical constituents of almost all members of the Bacteria. The Sherlock MIS system (MIDI Inc.) provides a comprehensive database, but this is certainly not complete and there are some discrepancies that need to be clarified or compounds that are currently not included in the database. When determining the fatty acid patterns of strains, the cultivation conditions of the strains should be identical prior to fatty acid extraction.

B. Analysis of Polar lipids
There is a vast diversity of polar lipids now known to be present in prokaryotes and in many cases their structures have yet to be fully elucidated. There is currently no collective work that adequately covers all aspects of prokaryote lipids, although the work of Ratledge & Wilkinson (1988) is a good starting point. Their biosynthesis is also not fully understood. It should be emphasized that the polar lipid diversity is associated with the cell membrane(s) and is not limited to just phospholipids. Given the large diversity, it is important to document the lipids present by providing an image of the thin layer plate stained with a reagent that will allow all lipids to be visualized. The range of polar lipids known to occur in members of the Bacteria is currently known to include phospholipids, glycolipids, phosphoglycolipids, aminophospholipids, amino acid derived lipids, capnines, sphingolipids (glyco- or phosphosphingolipids) and also hopanoids.
C. Analysis of Respiratory lipoquinones

Respiratory lipoquinones are widely distributed in both anaerobic and aerobic organisms within the *Bacteria* and *Archaea*. These may be divided into two basic structural classes, naphthoquinones and benzoquinones. A third class includes the benzothiophene derivatives, such as sulfolobus quinone and caldariella quinone, but data available to date indicate that this class is restricted to members of the order Sulfolobales (Tindall, 2005). Respiratory quinones which belong to a class of terpenoid lipids are constituents of bacterial plasma membrane and are valuable in microbial systematic (Collins, 1985).

1.4 ANTIBACTERIAL ACTIVITIES

1.4.1 Antibiotic resistance

The antibiotic resistant bacteria are increasing worldwide (Levy & Marshall, 2004; Hawkey, 2008). The rise of antibiotic resistant pathogens such as beta-lactams (β-lactams), methicillin-resistant and vancomycin-resistant has increased morbidity and mortality associated with bacterial infection and made effective treatment a significant challenge (Rice, 2006; Amyes, 2007; Reik et al., 2008). Costs associated with antibiotic resistant bacterial infections include increased patient care and treatment expenses and the need for newer and higher-priced antimicrobials to treat the most resistant infections (Hawkey, 2008). Unfortunately, at the same time the number of new antibiotics being approved and introduced into the market has steadily declined during recent decades (down 54% from 1983 to 2002) (Spellberg et al., 2004; Tenover, 2006; Taubes, 2008). Clearly, better management of antibiotic resistance is needed, as is enhanced knowledge of the ecology of antibiotic resistant strains and associated resistance genes. Exposure of bacteria to antimicrobials can select for those microbes that are intrinsically resistant, have acquired antibiotic resistant genes, or have a mutation that makes them resistant. The pressure applied to the microbial population by the antimicrobial eliminates the susceptible strains, leaving behind the resistant cells and over time the resistant microbes predominate (Levy, 2002; Hawkey, 2008). Two such environments where antibiotic selection pressure is intense are the clinical, such as hospital intensive care units (ICU) and agricultural settings related
to food animal production. Hospital ICUs are an ideal environment for the emergence, proliferation and maintenance of antibiotic resistant bacteria. Patients receive extended treatment with the majority being administered multiple broad spectrum antibiotics (Iredell & Lipman, 2005). Certain bacteria have adapted to this environment and established nosocomial strains that pose a significant challenge both for treatment and containment (Hawkey, 2008). For instance, the antibiotic resistance patterns of *Flavibacterium* and *Myroides* strains shows variable susceptibility to beta-lactams (β-lactams) (Holmes et al., 1979), there is constant decrease in susceptibility to cephalosporin and imipenem. The β-lactamases produced by gram negative and gram positive bacteria play a significant role in resistance against β-lactam antibiotics but with variable activities (Hummel et al., 2007; Mammeri et al., 2002). Strains of *M. odoratus* and *M. odoratimimus* are sources of nosocomial infections in humans and behave like low-grade opportunistic pathogens. *Myroides* spp. were identified as a cause of surgery wound and urinary tract infections, septicemia, pneumonia, meningitidis, fasciitis, and ventriculitis (Yagci et al., 2000).

As Holmes (1979) stated, “The problem of treating infections caused by gentamicin-resistant *Flavobacterium* spp. remains unsolved” looks suitable and valid statement. As a result of the wide variation in antimicrobial susceptibility shown by different species, a test on susceptibility to different antibacterial agents is essential in order to select an adequate therapy. The marked multiple drug resistance evidenced in some species, prompts the need to develop new antimicrobial agents active against this group of bacteria and to search for synergistic combinations. Nosocomial bacteria such as *Flavobacteria* are resistant to a wide range of antimicrobial agents like gentamycin, tobramycin, amikacin and carbenicillin to which Gram-negative, non-fermentative bacteria are expected to be susceptible to. This suggests that any infections due to these species would prove difficult to treat; in case of clinical cases involving systemic infections (Green et al., 2001; Holmes et al., 1979).
1.4.2 β-lactamases

The β-lactamases produced by Gram-negative and Gram-positive bacteria play a vital role in resistance against β-lactam antibiotics (Sato et al., 1985). Mammeri et al. (2002) proposed that although Myroides spp. possess metallo β-lactamases, it is difficult to predict the role of intrinsic β-lactam resistance, since metalloenzymes expressed in E. coli give much lower level of β-lactam resistance as compared to original producers. Such phenomenon is observed due to combined biosynthesis of carbenem derivatives and carbenem hydrolyzing β-lactamases as observed in Streptomyces sp. Gut bacteria inhibiting lepidopterans are known as producers of biosurfactants and act as plant volatiles, their exact role is not yet understood, but it is hypothesized that they might have same role as in case of mammals (Maneerat et al., 2006). Increased antibiotic resistance in such bacteria where β-lactamase gene subunits are lacking is mediated by acquiring resistance mechanisms through mutations in bacterial genome or by gaining additional genes through horizontal gene transfer or by physiology dependent resistance. Moreover, bacteria have intrinsic resistance mechanisms that are often not detected in standard antibiotic sensitivity tests performed at laboratory level (Saidijam et al., 2006). However, Myroides spp. are linked with low virulence and mortality when it comes to clinical level.

1.5 AIMS AND OBJECTIVES OF STUDY

1.5.1 Background

The study of microbial ecology of the insect gastrointestinal tract is experiencing a revival due to the development of molecular techniques for studying complex microbial communities. Prior to our study, knowledge of the house fly-associated microbiota was limited to culture-dependent assays (employing culture alone or culture followed by PCR) and focused only limited or even single species (Nayduch et al., 2001; Szalanski et al., 2004; Alam & Zurek, 2004). However, it has been suggested that less than 1 % of bacterial species can be cultivated (Staley and Konopka, 1985), and thus culture-dependent studies of house fly microbial ecology may be insufficient at best. However, there is a lack of studies characterizing bacteria housed in alimentary tract of the house fly. Therefore, in
this study we planned to characterize the total microbiota associated with the gut of house flies collected from various public places that represented both sanitary and unsanitary areas. These sites included a garden, public park, garbage dump, public toilet, hospital, restaurant/canteen, mutton shop/market and house/human habitation. We utilized both culture-dependent (i.e. live culture followed by 16S rRNA gene sequencing) and culture-independent (i.e. total DNA extraction, PCR, sequencing of cloned 16S amplicons) approaches (Pidiyar et al., 2004).

Despite the great medical, forensic and economic importance of flesh flies, the nature and diversity of the microbial communities housed in alimentary tract of flesh fly larval and adult guts has not been investigated. Many previous studies related to endocrinology, diapause, cold hardiness, reproduction, and immunity and genomic or transcriptomic ESTs (Hahn et al., 2009) have greatly enhanced our understanding of flesh flies in the genus Sarcophaga. However, there is lack of information about microbiota that is associated with the alimentary tract of the flesh fly. No studies so far have shed light on the total bacterial diversity associated with the guts of the flesh fly. In this study, we employed both culture-dependent and culture-independent (16S rRNA gene based sequencing) methods to characterize the total bacterial diversity associated with the larval and adult stage guts of the flesh fly, Sarcophaga sp.

The 16S rRNA gene sequences generated by both methods revealed a wide variety of new and unreported potentially pathogenic bacteria associated with the gut of flies. Results of this study can shed light on the possible role of flies as both vectors and environmental reservoirs for human pathogenic bacteria. The novelty of isolates or phylotypes found in the fly guts was expected, based on the insect’s physiology, the micro-environment in which these gut bacteria reside and also due to the low number of related studies. A polyphasic approach was used to characterize putative novel isolates found during the survey of gut bacteria based on 16S rRNA gene sequences.
Antibiotic resistance is the capability of bacteria and other microorganisms to resist the action of antibiotics. Multidrug resistance has become a serious issue in clinical medicine. The main concern is the increase of resistance development in virulent bacterial strains and its correlation to the heavy use of antibiotics. The resistant bacteria are selected for by the selective pressure and by disseminating and sharing the resistance genes (Bogaard et al., 2000). Flesh flies (Diptera: Sarcophagidae) are a well known causative agent of myiasis and their gut bacteria have never been studied for antimicrobial activity against bacteria. We have investigated antibacterial activities of selected isolate of multidrug resistant bacteria.

1.5.2 Objectives
The specific experimental aims and objectives of entire study were:

1. Molecular phylogenetic characterization of bacteria associated with the gut of house flies (*Musca domestica*)
2. Molecular phylogenetic characterization of bacteria associated with the gut of flesh flies (*Sarcophaga* sp.)
3. Polyphasic taxonomic approach to characterize two novel bacterial strains
4. Antibacterial activities of multidrug resistant bacteria