AN ABSTRACT OF THE THESIS

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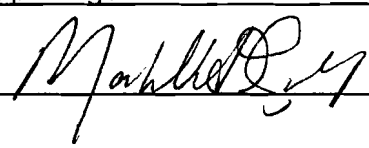
Title: The potential for house cricket, *Acheta domesticus*, to acquire, harbor and transmit human pathogens.

Abstract approved: 

Throughout human history, arthropods have served as the major group of vectors to transmit human pathogenic agents. Cockroaches are one of the most suspicious insects, transmitting many pathogens that cause diseases in hospital environments and living areas with poor hygiene. The house cricket, *Acheta domesticus*, shares similar gut morphology and feeding behaviors with cockroaches and also are closely associated with humans' habitation. However, it has not been reported that house crickets can acquire, harbor and transmit human pathogens not naturally present in their hindguts. In this project, *Serratia marcescens, Salmonella typhimurium, Echerichia coli* O157:H7 *and Bacillus atrophaeus* were chosen to inoculate house crickets to detect their ability to acquire and harbor these microorganisms. House crickets were able to acquire these four bacteria through their diet. However, only *S. marcescens* was carried by house crickets for as long as 18 days after

being inoculated. House crickets were not able to harbor either *S. typhimurium* or *B. atrophaeus* longer than 6 days. Moreover, there was only one cricket, being inoculated for 4 days, able to carry *E.coli* O157:H7. In conclusion, house crickets are less likely than cockroaches to harbor and disseminate human pathogens. There is little evidence for relating human diseases to house crickets.

THE POTENTIAL FOR HOUSE CRICKET, *ACHETA DOMESTICUS*, TO ACQUIRE, HARBOR AND TRANSMIT HUMAN PATHOGENS

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PREFACE

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Introduction

Vectors and vector-borne diseases. Worldwide, human infectious disease is the most common cause of death. In the United States, infectious disease is the third leading cause of death. Many of these infectious diseases are transmitted by vectors. The mosquito is perhaps the best known vector, but many other insects also serve as vectors, and yet others are purported to have the potential for transferring an infectious agent to humans. In the United States, vector-borne diseases are becoming a growing concern since an estimated 56% of households contain at least one pet, which may host a variety of arthropods (Glaser et al., 2000).

Based on the relationship between vectors and pathogen, vectors can be categorized as biological, harborage or mechanical. By serving as a biological vector, the vector's body provides a habitat which facilitates completion of the life cycle of a pathogen. During this time, the vector might feed on humans and transmit the pathogen through saliva or feces into a break on the skin (Leclercq, 1969). On the other hand, certain vectors simply harbor pathogens within their bodies. (These pathogens do not undergo morphological or physiological change.) Hence, these vectors are called harborage vectors. Similar to a biological vector, they transmit the pathogen through saliva or feces. A third category is called a mechanical vector. The vector becomes contaminated with a pathogen on its external body parts. It subsequently transfers this pathogen to a human either directly or by an intermediate like food.

Throughout human history, arthropods have served as the major group of

vectors for transmitting human pathogenic agents (Nester et al., 2003). Moreover, arthropod vectors are found in each of the vector categories listed above. Examples of arthropod vectors follow.

The sandflea is a biological vector. It transmits an obligate intracellular protozoan of the genus Leischmania. First, the sandflea ingests *Leischmania* when it takes a blood meal from an infected animal. Within the sandflea, *Leischmania* completes another stage of its life cycle and multiplies. Next, when a human being is bit, *Leischmania* are transmitted to the skin. The human body responds by engulfing the protozoan inside a macrophage; a type of human white blood cell. In some individuals, replication and metamorphosis or parasites within macrophages is responsible for the disease, Leischmaniasis.

With some exceptions, human beings serve as incidental hosts of infection, and other mammals serve as reservoir hosts. Because parasite replication may occur in macrophages in the mononuclear phagocyte system, dermis, and/or naso-oropharyngeal mucosa, the names visceral, cutaneous, and mucosal leshmaniasis respectively are applied to several clinical syndromes. If untreated, life-threatening systemic infection can be caused by visceral leishmaniasis (Herwaldt, 1999).

The ixodid tick is a harborage vector. It transmits a spirochete called *Borrelia burgdorferi*, the cause of Lyme's Disease. The larval and nymphal stages of these ticks prefer to parasitize on the white-footed mouse *Peromyscus leucopus*. The white-tailed deer *Odocoileus virginiamus* serves as the host for the adult tick. When the ticks feed in autumn, the infected ticks occasionally transmit the

pathogen by biting people. Erythema migrans (i.e., a skin infection that spreads) appears in the early infection. The spirochete spreads to different sites of the human body causing symptoms involving multiple organ systems throughout the body, such as skin lesions, meningitis, facial palsy, radiculoneuritis, atrioventricular nodal block, or migratory arthritis in joints (Steere, 1994).

Another harborage vector is the rat flea, *Xenopsylla cheopis*. It transmits *Yersinia pestis*, the etiological agent of bubonic plague (Prescott et al., 2002). Rodents are the major reservoir of *Yersinia pestis*. When the rat flea feeds on the rodent, it ingests viable *Y. pestis* cells and then multiplies in the intestinal tract. By biting other animals like humans, the rat flea transmits *Y. pestis* to the next host. Human body temperature, about 37°C, is optimal for this organism to grow and produce F1 and VW antigens as its virulence factors, which keeps *Y. pestis* from being killed by polymorphonuclear leukocytes and allows them to travel the lymph nodes. The black buboes of "bubonic" plague are caused by the inflammation and hemorrhage of lymph nodes. About 50-60% of untreated individuals die of severe bacterial pneumonia (Nester, 2002).

The housefly *Musca domesticus* can serve as either a harborage vector or a mechanical vector. As a mechanical vector, the housefly might transmit diarrhea-causing bacteria such as *Salmonella* (Olsen and Hammock, 2000), *E. coli* 0157 (Sasaki et al., 2000), *Vibrio cholerae* (Fotedar, 2001), and *Shigella* (Levine and Levine, 1991). With regard to *Shigella*, certain species of the genus *Shigella* are responsible for Shigellosis, also known as bacillary dysentery. Symptoms like bloody diarrhea, fever and stomach cramps appear a day or two after being

exposed to the pathogen. *Shigella* are gram-negative, rod-shaped bacteria which infect the human intestinal tract. The housefly contacts *Shigella* via infected human feces and transfers the bacteria by subsequently touching human food (Levine and Levine, 1991). According to results obtained by Cohen et al., 1991, in two military bases in Israel, the diarrheal infections of cohorts were significantly reduced by the control of houseflies, indicating that houseflies serve as mechanical vectors to transmit *Shigella* diarrheal infections (Cohen et al., 1991). Besides *Shigella*, houseflies are also suspected of transmitting *Salmonella* (Olsen and Hammock, 2000), *E.coli* O157: H7 (Sasaki et al., 2000), *Vibrio cholerae* (Fotedar, 2001) and *Helicobacter pylori* (Brown, 2000). However, two conflicting studies share the different opinion for the transmission of *H. pylori*. In the study by Grubel et al. (1997), houseflies were demonstrated to be able not only to harbor viable *H. pylori* on their bodies and in their intestinal tracts, but also to deposit viable *H. pylori* in excreta, which implies that houseflies may serve as a reservoir and a vector in the transmission of *H. pylori*. On the contrary, the study by Osato et al. (1998) showed that viable *H. pylori* couldn't be recovered from the feces of houseflies, indicating that houseflies may not be able to harbor and transmit *H. pylori* to humans.

Cockroaches are known to serve as harborage and mechanical vectors of human pathogenic agents (Bennett, 1993; Ramirez, 1989). They are closely associated with humans' habitation where they reproduce prolifically wherever leftover food is available. The American cockroach, *Periplaneta Americana,* and German cockroach, *Blattella germanica* are the most common cockroaches

caught and studied. According to studies conducted in different countries during the past thirty years, most potential human pathogens were isolated from cockroaches collected from hospitals (Pai et al., 2003; Oothumanet et al.,1989), sewage and residential areas with poor hygiene (Cloarec et al., 1992). A large micro biota is associated with the chitinous gut wall and with the external body parts. Bacterial colonies which cause disease in humans also were recovered from gut contents and fecal specimens of cockroaches collected in nature and in the laboratory (Paul et al., 1992). It is therefore likely that cockroaches serve both as mechanical vectors and harborage vectors for disease transmission. Bacterial species isolated from cockroaches include *Klebsiella* spp., *Mycobacteria* spp. *and Pseudomonas* spp. These are nosocomial pathogens (i.e., hospital acquired) and cause pneumonia, tuberculosis and surgical infections respectively (Rivault et al., 1993; Pei et al., 2003). An outbreak caused by extended spectrum beta-lactamase-producing *Klebsiella pneumoniae* in a neonatal unit infested with cockroaches in South Africa suggested that cockroaches are possible vectors of pathogenic bacteria in the hospital environment (Cotton et al., 2000). Recently, drug-resistant bacteria were isolated from some collected cockroaches. The medical importance of cockroaches should be noteworthy in the future (Devi and Murray, 1991; Fotedar et al., 1991).

The house cricket. House crickets, *Acheta domesticus*, are another arthropod highly active in human environments. Moreover, their gut morphology and feeding behavior are similar to those of cockroaches. Based on an extensive literature search, it appears that there is very little known about the cricket as a

vector for human pathogens. Thus, in the present study the house cricket was chosen to be tested for the potential of harboring and transmitting human pathogens. The house cricket is an insect having three main body parts, six legs and long antennae. Its leathery front wings are folded over the side of their body and two long hind legs are fitted for jumping. Two cerci extend out from the abdomen of both sexes. Unlike a field cricket's blackish body, house crickets are straw-colored. There are some banding and spotting in brown around its body, such as a brown band back of the eyes and between the eyes and around the antennae, and brown spots on the thorax (Stone, 1953).

A two week old young cricket's body is about 5 mm in length. A fully developed adult may grow up to 2.5 cm long. Its antennae may extend to be as long as its body. Crickets demonstrate gradual metamorphosis, which is an important characteristic of the *Orthoptera*. There are only three stages from egg to adult cricket during the process of development. The tiny nymph, hatching from the egg, is the only intermediate stage and looks like an adult cricket, but wingless. Only adult crickets have fully-grown wings (Woodring, 1983). To distinguish the females from males, the easiest way is to check for an ovipositor. Adult females use this needlelike organ, extending from the abdomen and between the two cerci, to push eggs into the ground while laying. A female cricket may produce about 2,600 baby crickets during her life-span (Stone, 1953).

During the mating season, males use their scrapers and files formed by the ridges on the front wings to make a chirping call to attract the females and claim their territory (Popov, 1971) . To hear the chirping and locate the rnales, females

use a small pit covered by a thinly stretched membrane on the front side of their forelegs as their ears (Nelson et al., 1997).

House crickets like to stay in warm places and the most comfortable temperature for them ranges from 27°C to 32°C. Therefore, house crickets tend to get into houses or hide in the basement to keep warm through the cold winter. Temperature is the leading factor that influences young crickets to develop. Nymphs take fewer days to mature while kept at 32°C than 27°C.

Crickets are omnivorous. A great variety of foods can serve as their food source, such as fruits, vegetables, all kinds of meat, and fabric materials. It's not very difficult for crickets to find food in nature. Sometimes, crickets will eat fresh leaves of vegetables to get moisture. Crickets are also cannibalistic. When food is deficient, crickets may eat each other to survive (Roe et al., 1980).

Crickets have a lot of enemies in nature. Some flies can hear the male crickets' calling, locate it, and deposit larvae on the calling male. These tiny larvae can bore through the exoskeleton of this male and live and grow inside the host's body until they are ready to pupate. Moreover, any kind of animal that eats meat and can catch a cricket, like frogs, toads, snakes, birds, and even animals like a fox, skunk, and raccoon, might be a cricket's natural enemy. Meanwhile, crickets may be an enemy for each other under certain circumstances. For people who love fishing, crickets serve as a good and abundant source of fishing bait (Stone, 1953).

Crickets share the same habitat with human beings and are used and sold commercially in various human activities. Because they are highly active around

human households, it is very likely for crickets to contact human pathogens with their external parts of body. And because they are omnivorous, it also is very likely that they ingest food, water and sewage, which may be infected by human pathogens.

Ulrich et al. (1981) studied the intestinal flora in farm-raised crickets and isolated some harmless species of intestinal bacteria. Another study specifically looked for aerobic and anaerobic human pathogens in the hindgut of both house crickets and field crickets. Many human opportunistic pathogens were naturally present in the hindgut (Smith and Gaeddert, 2002). To my knowledge, it has not been reported that house crickets can acquire, harbor, and transmit human pathogens which are not naturally present in their hindgut.

Microorganisms. In the present study, three human pathogens were selected: *Serratia marsescens, salmonella typhimurium* and *Escherichia* coli 0157. I assessed the potential for the house cricket to acquire, harbor and transmit each pathogen. A fourth species, Bacillus atrophaeus, was selected for its similarity to Bacillus anthracis, which is too virulent for a direct study. Similar to the other species, B atrophaeus was evaluated for its potential to be transmitted by crickets to humans. A description of each species follows. Serratia *marcescens*, a Gram-negative bacillus and member of *Enterobacteriaceae*, is able to utilize a wide range of nutrients and survive under extreme conditions. Therefore, it is widely distributed in water, soil, sewage, food and even in disinfectant. The production of prodigiosin forms red colonies on the surface of starchy foods. It was mistaken for bloody drops on Eucharistic bread, which was thought to

symbolize the conversion of the bread into the body of Christ in many historical reports (Yu, 1997). From the beginning of the last century, this organism was utilized as a biological marker in many experiments investigating the dispersal of bacteria by different means (Yu, 1997). *Serratia marcescens* is recognized as a nosocomial opportunist and an important pathogen with resistance to some antibiotics. It rarely causes disease in healthy persons, but it can cause pneumonia or septicemia in immune-comprised hosts (Hejazi and Falkiner, 1997). The colon of vertebrates is one of the main reservoirs of this organism. Therefore, *S. marcescens* is also found in fecal contaminated water supplies and sewage. Because crickets share the same habitat with human beings and other animals, it is likely that they acquire and transmit this pathogen.

Salmonella typhimurium, a member of Enterobacteriacea, is one of the most studied strains of *Salmonella* with more than 2,300 serotypes identified during the past 15 years. It is widely distributed in a variety of reservoirs in nature ranging from livestock, poultry, and reptiles to pets in households (Gomez et al., 1997). It is a true pathogen. Consumption of unpasteurized dairy products, undercooked contaminated meats and poultry products are the major routes of exposure to this organism (Villar et al., 1999). There are also reported cases of infections acquired by touching and ingesting feces from infected cattle on a ranch and infected cats in veterinary facilities (Fey et al., 2000; Ezell et al., 2001). The clinical course of human salmonellosis caused by *S.typhimurium* is usually characterized by acute onset of fever, abdominal pain, diarrhea and vomiting. The symptoms in healthy persons are mild and self-limiting. For young children,

the elderly, and immune compromised persons, gastroenteritis is severe and may lead to life threatening dehydration. It also causes endocarditis and osteomyelitis in patients with sickle cell anemia (Schaehter et al., 1998). With extensive use of antibiotics in developed countries, there is a dramatic rise of multidrug-resistant strains. The definitive phage type 104 (DT104) is the most studied strain (Glynn et al., 1998; Molbak et al., 1999) There have been numerous cases reported in west European countries and United States since it was first isolated in 1988 in England and Wales (Gomez et al., 1997). DT104, which is resistant to ampicillin, chloraphenicol, streptomycin, sulfonamides and tetracycline, has become a widespread pathogen in our living environment and accounts for approximately 30% of all S. *typhimurium* infections in U.S. (Villar et al., 1999). Sewage and feces from infected humans and animals might also contaminate water supplies, and human households may be shared by house crickets. With the activities of house crickets around the human household, *S. typhimurium* might be carried and transmitted by crickets.

Echerichia coli O157:H7, a member of *Enterobacteriacea*, causes enterohemorragic colitis in humans. It is a true pathogen (Mead and Griffin, 1998). Two major toxins, shiga toxin 1 and shiga toxin 2, are responsible for the symptoms (Takeda, 1997). The normal course of infection includes watery diarrhea turning into bloody stools. Serious infection can lead to a particular form of hemolytic anemia, thrombocytopenia, and renal failure. Complications can cause the death of young children (Boyce et al., 1995). About 1 % of healthy cattle carry this organism in their intestinal tracts. In human infections, it is likely

that beef becomes contaminated during the process of food production. In the United States, it is estimated that 20,000-40,000 infections are caused by *E.coli* O157 :H7 yearly. Consumption of raw or undercooked contaminated meat products is associated with most of the outbreaks (Mead and Griffin, 1998). Contaminated water supplies for some field crops also serve as a source of infection. Unpasteurized apple cider and contaminated raw vegetables were suspected as the source of infection in some sporadic outbreaks (Takeda, 1997). House crickets might become infected with *E.coli* O157:H7 by touching or eating the contaminated water and food around human households.

Bacillus atrophaeus, a harmless spore-forming Gram-positive bacillus, was used to assess the potential for a house cricket to acquire and harbor a spore producing organism. Because of its ability to produce spores of specific resistance to dry heat or ethylene oxide, it was specially suited for testing the effectiveness of methods for sterilization (Fritze and Pukall, 2001). *B. atrophaeus* has been used by the U.S. Defense Department as a model for studying *Bacillus anthracis*, an etiological agent of the fatal pulmonary anthrax (Weber, 2003). If the environment is contaminated by the *B. anthracis* spores, insects might serve as an incubation chamber for germination and then reproduction. Therefore, it is possible for crickets to further spread the pathogen in the environment through their feces. Because of the threat of terrorist attacks using *B. anthracis* spores as a weapon, the potential for crickets to acquire spores and deposit feces with spores or spore-forming vegetative cells was investigated.

In this study, the house cricket was evaluated both as a possible harborage

vector, by multiplying the pathogen in the intestinal tract and depositing it in the feces to transmit human pathogens; and as possible mechanical vector, by touching the sewage and foods from human households with the outer parts of their body to transmit human pathogens.

In a preliminary study, we examined methods of inoculating crickets, either via drinking water or injection through their mouth. We also tested the ability to successfully recover *S. marcescens* colonies from both the cricket hindgut and feces for as long as 120 hours after inoculation. Finally we tested house crickets with these above three human pathogens and one spore-forming organism for their ability to acquire, harbor and transmit them by feeding. Bacteria were recovered and identified using specific agar plates and tests.

Materials and Methods

Inoculating and collecting samples from the hindgut of house crickets. House crickets, *Acheta domesticus*, were purchased from a local pet store (L & L Pets, Emporia, Kansas). Prior to inoculation, crickets of similar size were kept in individual sterile glass jar (Fig. 1 A) and supplied with sterile water and feed (Purina Chicken Feed). Fecal samples from each cricket were tested for the presence of each pathogen used in this study. According to the results of the testing, crickets free of a particular pathogen were randomly assigned to either a control group or a test group for that microbe. Crickets for both test group and control group were housed separately in sterile Pyrex storage dishes (Corning #3250 100) (Fig. 1 B), designated as crickets' cages. Throughout the study, all of the control groups were supplied with sterile water and feed.

In a preliminary study, we ran a test for the viability of *S. marcescens* (Fisher Scientific S20937) in drinking water at 1, 2,and 3 days after inoculation. We recovered *S. marcescens* colonies on Tryptic Soy Agar (TSA, Difco, Detroit, MI) after all three time periods. We also tested direct injection (Fig. 2) as a means of inoculation to avoid the possibility of mechanical transmission. We conducted a series of tests to determine the optimal dosage for injection. Crickets were injected with 10µl of inoculum at the following dilutions: 10^3, 10^4, 10^5, 10^6 and 10^7 bacteria/ml. The test groups in the studies of *Serratia marcescens* and *Salmonella typhimurium* (ATCC13311) were provided with sterile feed, but the supplied water was inoculated with pathogens at the concentration about 6×10^8 bacteria/ml for three days. For *Bacillus atrophaeus* (Bacillus Genetic Stock

Center BGSC# 12A1), the test groups were provided with sterile water. The supplied feed was mixed with spores from colonies of *B. atrophaeus* for three days. A pair of crickets, one from the control group and the other one from the test group, were randomly picked up and dissected at the end of the third day, defined as day 0. The remaining crickets were transferred to a new sterile cage and supplied with sterile feed and water. At three days intervals, this procedure was repeated for as long as the remaining crickets survived.

Because of the biosafety concerns with the vicious enterohemorragic *E.coli* (EHEC), I assisted Dr. Gaeddert in investigating the ability for crickets to harbor this pathogen. To reduce the risk to the investigator, two different strains were used. A fully virulent strain (ATCC43894) containing both Shigella-like toxin1 and toxin 2 was used only to examine whether the crickets would become ill or die. A strain not carrying the genes for toxin 1 and 2 (ATCC43888) was used to determine if the crickets would harbor this pathogen in the hindgut. Ten crickets for the control group and 20 crickets for the test group were housed in two separate cages for testing the ability for acquiring and harboring *E.*coli 0157:H7. The control group was provided with sterile water and feed throughout this experiment. The test group was inoculated with this pathogen through drinking water at 9×10^8 bacteria/ml for 3 days. A fresh inoculum was placed in drinking water each day. At the end of the third day, defined as the day 0, two crickets from the control group and four crickets from the test group were randomly selected and then dissected. Each day, the remaining crickets were transferred

to new sterile cages and were supplied with sterile feed and drinking water. Dissections were performed every day for seven days.

Dissection. In preparation for dissection, crickets were sacrificed by placing them in the freezer for 15 minutes followed by disinfection in 90% alcohol. (The effectiveness of disinfection of the surface of the cricket was evaluated by disinfecting bacteria-coated glass beads in 90% alcohol and then placing the beads in sterile broth. For each species, evidenced by the absence of growth in the brother, disinfection had killed all bacteria on the surface of the glass beads.)

Using aseptic technique (i.e., sterile pins, scissors, forceps and dissecting pan), the disinfected cricket was removed from the alcohol, placed on the dissecting tray and then beheaded. After the neck was pinned, and incision was made from a point anterior and ventral to the anus through the abdomen and thorax. Sterile pins were used to tease open the abdominal cavity and expose the gastrointestinal tract (Fig. 3). Then, the hindgut region was lifted away from the abdominal cavity by using sterile forceps and the gut was cut at the most proximal end (where it joins with the stomach) and at the distal end (just proximal to the anus). Then, the hindgut sample was emulsified in sterile saline and applied onto appropriate media and onto slides for identifying the particular pathogen. Two plates were made from each sample.

A total of four replicates were run for *S. marcescens* and the data was collected and combined for these experiments. Two and three replicate experiments were run for *S. typhimurium* and *B. atrophaeus*, respectively. Due to time constraints, a single experiments was run with *E. coli* 0157:H7.

Identification of the pathogen in the hindgut. Serratia marcescens: This pathogen produces a red colony on general media. In this study, the hindgut sample collected from cricket's intestinal tract was inoculated onto a TSA plate. It was easy to identify the presence of *S. marcescens* among the flora of the cricket hindgut (Fig. 4 A, B and C). In addition, the Gram stain was used to verify whether the red colonies were comprised of Gram-negative rods (Fig. 5).

Salmonella typhimurium: This pathogen produces blue-green colonies with black centers from hydrogen sulfide gas on Hektoen Enteric (HE, Difco) agar (Fig.6A). In this study, the hindgut sample collected from cricket's intestinal tract was inoculated onto a HE plate and incubated for 24 hours. To verify that the blue-green colonies with black centers were *S. typhimurium,* bacteria from the colony were inoculated into a pair of broth tubes. One tube contained lysine decarboxylase broth and the other tube contained decarboxylase broth. After 24 hours of aerobic incubation at 37^0C, this pathogen should turn the lysine decarboxylase broth deep purple and the decarboxylase broth yellow (Fig. 6 B) (Koneman et al., 1992). For further confirmation, a sample of bacteria from the blue-green HE colonies was inoculated into a tube of tryptophan broth (Difco) to incubate overnight. For testing the presence of tryptophanase, the KOVACS' Indole reagent (Merck) was used (also called the Indole test) (Koneman et al., 1992). With the Indole test, *S. tyhimurium* is indole-negative; a red-pigmented ring should not develop on the surface of a tube of tryptone broth(Fig. 6 C).

Bacillus atrophaeus: This bacterium produces a light brown colony on both TSA plates and Tryptone Yeast Extract (TYE, Difco) plates. In this study, the

hindgut sample collected from the cricket's intestinal tract was inoculated onto a TYE plate. It was very easy to identify the presence of *B. atrophaeus* on the plate after the incubation. To further confirm the identity, Gram-staining was used to verify that the brown colonies are comprised of Gram-positive rods (Fig. 7), and spore staining was also conducted for the hindgut samples. (Fig. 8). .

Echerichia coli O157:H7 (EHEC): To identify the presence of EHEC in the hindgut flora, MacConkey agar(Difco, Becton Dickson, Sparks, MD.) was used to recover this pathogen. Within this highly-selective and differential medium, crystal violet selects against Gram-positive organisms, and the lactose differentiates between lactose fermenters (red colonies) and non-lactose fermenters (colorless colonies). EHEC produces red colonies on MacConkey agar after being inoculated and incubated for a proper time. However, several other genera and all natural strains of *E.coli* share the capabilities to grow and ferment lactose on the MacConkey agar. To further differentiate among the strains of *E.coli* and confirm the presence of EHEC in the hindgut suspension, red colonies were applied onto the modified MacConkey plate containing polyalcohol, sorbitol, instead of lactose. The EHEC is the only strain unable to ferment sorbitol among the all strains of *E.coli*. It produces colorless colonies on the modified MacConkey plate (Fig. 9 A: sections 6B, 8BX, 2). To differentiate among the other lactose-fermenting, sorbitol-fermenting Gram-negative genera, colorless colonies were tested for indole production. All *E.coli* strains are indole positive (Fig. 9 B left). A lactose-fermenting, sorbitol non-fermenting, indole-positive isolate from the hindgut of the house cricket might be EHEC. To ensure

the presence of the EHEC strain in the sample, a latex agglutination kit purchased from Pro-Lab Diagnostics was used to perform a serological test specific for this organism. The identity of the bacterial isolate was confirmed to be EHEC when there was agglutination with the anti-EHEC-coated latex beads(Fig. 9 C) (Koneman et al., 1992).

Results

The preliminary experiments. In the preliminary test, we inoculated crickets with *Serratia marcescens* in two ways to evaluate their potential to carry and deposit viable bacteria in their feces. The production of prodigiosin by *S. marcescens* colonies allowed us to distinguish them easily from other bacterial colonies that appeared on TSA plates because of the production of red pigment. Initially we inoculated crickets through drinking water with 100 µl of inoculum of *S. marsescens* in 2 ml of sterile distilled water. The results are summarized in Table 1. Red colonies formed on the TSA plates from the hindgut and fecal samples at 32, 40, 48 and 56 hours after incubation. None of the TSA plates from both the hindgut of 16 and 64 hours after incubation demonstrated red colonies, but there were red colonies on the corresponding TSA plates from both fecal samples. None of the 24 hours post incubation plates from either the hindgut or feces showed the presence of viable *S. marcescens.* The red colonies that formed on the TSA plates were subcultured to further confirm their identity as *S. marcescens.*

To ensure that the bacteria were ingested into the crickets' gastrointestinal tracts as opposed to being mechanically transmitted, we inoculated a series of crickets with *Serratia marcescens* by injecting inoculum into their esophagus. Some of the crickets injected with a high dose of 10^7 bacteria/ml, died and their entire bodies turned red (Fig. 10). Some crickets injected with low dose such as 10^3 bacteria/ml and 10^4 bacteria/ml either died and turned black or viable *S. marcescens* was not recoverable from their hindgut. (Table 2). After several

repetitions, we decided that 10µl of 10^6 bacteria/ml, 700-1100 bacteria per cricket, was the optimal dose for injection.

We collected samples from hindguts and feces at 8 hour intervals after injection for two days. Except for the 8- hour group, we were able to recover the red colonies on TSA plates from all hindgut samples (Table 3). However, we did not recover any red colonies on the TSA plates from the fecal samples, indicating no viable *S. marsescens* existed in these feces. We also dissected crickets at 72, 96 and 120 hours after injection. All of the TSA plates exhibited *S. marsescens* colonies from the hindgut samples, but only the 96 and 120 hours post injection crickets produced feces with viable bacteria (Table 3).

Viablity and harboring experiments. The colonies of *S. marcescens* were recovered on the TSA plates from 3, 6, 9, 12, 15, 18 days after the first day of incubation (Table 4, Fig. 4). In an extensive experiment, the house cricket was demonstrated to survive for 20 days and *S.marsescens* colonies were also recovered from its hindgut sample. To confirm the identity of this pathogen, Gram stain slides were made from the subcultures of these colonies (Fig. 5).

In the study of *Salmonella typhimurium*, crickets were also inoculated through their drinking water and kept in the same condition as described above. Initial colonies were cultured onto HE plates. To further confirm that the blue- green colonies were *S. typhimurium* a subculture was streaked onto another new HE plate. One day later, the colonies were inoculated to run the indole test and lysine decarboxylase test. As a result, the blue-green colonies with black centers of *S. typhimurium* were recovered on HE plates from 3 and 9 days after the first

day of incubation (Table 4). The results of indole test and lysine decarboxylase test also confirmed these colonies to be *S. typhimurium*. The Indole test for *S. enteritidis* should be negative as identified by no red -pigmented ring on the surface of the tryptophan broth (Fig. 6 B right). The colonies recovered from the crickets of 6 days after incubation showed blue-green colonies with black centers on HE plates, and the lysine decarboxlase test also showed the positive reaction; however, the results of the Indole test was not consistent with *S. typhimurium*. After we ran several tests and checked with the available reference, we still couldn't confirm the identity of these colonies. The rest of crickets survived for at least 15 days, but we couldn't recover any *S. typhimurium* colonies from their hindguts.

The *Bacillus atrophaeus* colonies were recovered on the TYE plates from the crickets on 0 day and 6 days after inoculation (Table 4, Fig. 11). More colonies formed on the 0-day plates than on the 6-days plates. However, no colonies were isolated from the hindgut samples of the crickets at 6-days after incubation. These crickets survived at least 15 days, but no colonies were recovered from these crickets' hindguts. To further confirm the presence of these spores in the crickets' hindguts, a spore stain and a Gram stain were conducted. In the spore stain of hindgut samples from the 9-days cricket, there were spores shown on the slide (Fig. 8.) but no colonies recovered on the TSA plates.

Two strains of *Echerichia coli* O157: H7 were used in this study. Crickets inoculated with the fully virulent strain (ATCC43894) containing both Shigella-like toxin 1 and toxin 2 survived through the study a total of 10 days, indicating that

this pathogen did not cause disease in crickets. However crickets inoculated with the strain (ATCC 43888), not carrying the genes for toxin 1 and 2, were susceptible but only 1 out of 20 crickets in the test group at day 4 post inoculation was able to harbor viable *E.coli* O157:H7 in its hindgut. There were no *E.coli* colonies on MacConkey agar plates recovered from crickets of day 0, 1, 2, 3 post inoculation (Table 4). A series of tests, including the inoculation onto modified MacConkey agar, indole test and a serological test using the anti-*E.coli* O157: H7coated latex to agglutinate this bacteria, were performed to confirm this bacteria. Red colonies recovered on the MacConkey agar plates were inoculated onto a modified MacConkey plate. EHEC unable to ferment sorbitol showed colorless colonies on the modified MacConkey plate. An indole-positive reaction and an agglutination reaction with anti-EHEC coated latex beads further confirmed the identity of *E.coli* O157: H7.

Discussion

In a preliminary test, we inoculated crickets with *Serratia marcescens* via diet and injection through their mouths. In the first part of the preliminary test, house crickets were kept in an individual sterile cage supplied with sterile food and inoculated drinking water. Except for the crickets at 16-hours, 24-hours and 64-hours, all of the crickets dissected recovered red colonies on the TSA plates. It is possible that the lack of bacterial growth at any time period was simply a statistical anomaly. That is, viable bacterial may have been present in the gut or feces but we happened not to sample them. A larger sample size would allow this alternative to be tested. Alternatively, the results could be because crickets ingested more bacteria when they were exposed to the pathogenic source for a longer period of time. With longer exposure it is more likely that the bacteria could multiply in cricket's hindgut. Except for the 24-hours crickets, red colonies were recovered from the fecal samples of all crickets, suggesting that viable bacteria were able to pass through the intestinal tract and be deposited in feces. According to our results, we presumed that house crickets can acquire *S. marcescens* from their diet and deposit it into its feces.

In the second part of the preliminary test, we inoculated crickets with *S. marcescens* by injection through their esophagus to ensure that there were sufficient bacteria getting into cricket's intestinal tract. This also would control for mechanical transmission. In order to determine the optimal dose for the crickets, we ran a serial dilution at 10^7, 10^6, 10^5, 10^4, and 10^3bacteria/ml. We were able to recover the *S.marcescens* colonies in hindgut samples of crickets injected with

10^7bacteria/ml of bacterial suspension, but half of the crickets died and their entire bodies turned red. It appears that this high dose of *S.marcescens* was lethal, perhaps by promoting massive sepsis in these crickets. On the other hand, no red colonies were recovered from the hindgut of crickets injected with $10^4$and 10^3bacterial/ml. The optimal dose for injection was chosen to be 10^6bacteria/ml, because at this level a maximum number of viable colonies were recovered. Approximately half of the crickets survived and not all of them turned red.

After being injected, crickets were kept in individual cages provided with sterile feed and drinking water. Except for the 8-hours crickets, red colonies were recovered from the hindgut samples of crickets dissected at all time periods, indicating that *S.marcescens* was able to rnultiply and colonize in crickets' hindgut if they were given a sufficient inoculum. However, only crickets dissected at 4-day and 5-day were able to deposit viable bacteria into feces. The fact that bacterial colonies were produced from feces of both shorter and longer duration suggests that the absence of bacterial growth in 72-hours feces was a sampling anomaly.

In the preliminary experiments we demonstrated that house crickets possess the potential to harbor *S.marcescens* for as long as 5 days within their gastrointestinal tracts and to deposit viable *S.marcescens* into their feces. Nonetheless, the crickets, kept in the cages during the process also walked through their food and water and could therefore mechanically transmit the pathogens. Simply examining the feces would not indicate whether the feces

containing the pathogens passed out of the anus, or, the feces were contaminated mechanically. Therefore, in the viability experiments for these four microorganisms, samples were collected directly from crickets' hindguts only. Because of the high mortality of the injected crickets in the preliminary study and because inoculation via diet is the most likely scenario to occur in nature, we inoculated crickets with these four species of bacteria via either food or drinking water throughout the main study.

We conducted a test for the viability of *S. marcescens* in drinking water after 1, 2 and 3 days and successfully recovered many red colonies on TSA plates, implying that the crickets had a sufficient chance to become infected through drinking water three days after exposure to the inoculum. After 3 days, crickets were transferred to new sterile cages with sterile feed and water. To test the ability of crickets to harbor viable colonies of these pathogens, we collected and combined data from several repetitions of these experiments. *S. marcescens* survived in the cricket's hindgut for as long as 18 days after being ingested. The supplied dosage of *S. marcescens* was not a threat to the cricket's life and was sufficient to allow it to multiply and colonize the cricket's hindgut. Compared to the life-span for a cricket, about 6 to 7 weeks, these results suggest that crickets may be a vector for transmitting this pathogen, once they have a chance to ingest, acquire, and harbor *S. marcescens*. Throughout this period, house crickets also are likely to transmit this pathogen through its feces or by mechanical touch. *S. marcescens* has a very low nutritional need and is able to survive in extreme environments. It also is found in many treatment facilities in

hospitals (Hejazi and Falkiner, 1997). Therefore, it is one of the most reported and studied microorganisms associated with hospital-acquired diseases. A study regarding the control of flying insects that enter food establishments reported that *S. marcescens* was successfully ingested by houseflies and survived on and within fly corpses for up to 5 weeks after electrocution of the insect (Cooke et al., 2003). *S. marcescens* also was isolated from hindguts of cockroaches collected from nature and hospital environments (Prado et al, 2002). These studies support my findings that *S. marcescens* can exist in cricket's hindgut for more than 2 weeks after being ingested.

In the viability experiments with *Salmonella typhimurium* in house cricket's hindgut, we found that the test crickets only carried this pathogen in their hindguts for up to 6 days following 3-days exposure to the bacteria. Lack of viable colony production in our test crickets of 3-days was probably a statistical sampling error. We couldn't recover any blue- green colonies on HE plates from the hindgut samples of a test cricket in one repetition of the 3-days; we did recover blue-green colonies with black centers from a test cricket of the second repetition. However, in verifying the identity of these colonies, we inoculated pure cultures from these plates and ran a series of biochemical tests, including the lysine decarboxylase test, indole test, and KIA test, several times. The results from these tests met the criteria for *S. typhimurium,* but not the indole test. In spite of these efforts, we still couldn't confirm the identity of these colonies from the 3-days test crickets. These results suggest that during the cricket's average life-span under given conditions, *S. typhimurium* either cannot

compete with the normal flora or is not well adapted to the physiological conditions in cricket's gastrointestinal tract.

In the viability experiment with *Bacillus atrophaeus* in crickets' hindguts, the results we obtained were similar to the results from *Salmonella typhimurium*. We found more brown colonies on TYE plates from the hindgut of the crickets of the day 0, after being exposed to *B. atrophaeus* for 3 days, than day 6. Like *S. typhimurium,* we couldn't recover any brown colonies from any of the three repetitions at day 3. Nevertheless, we found spores in our spore stain slide from the hindgut sample of day 9. Several questions arise because of this circumstance. We couldn't be sure whether the brown colonies we found on TYE plates from the hindgut samples were recovered from: 1) viable *B. atrophaeus*, germinating from ingested spores in the hindguts, which then multiplied internally; 2) ingested spores that germinated and multiplied only after deposition on the TYE plates; or 3) some combination of the above. If future studies can recover *B. atrophaeus* colonies from cricket's feces, *via* viable bacteria or survived spores, it may be possible to definitively determine if crickets can acquire, harbor and transmit this microorganism.

In the viability experiment with *E.coli* O157:H7 (O157) in house cricket's hindgut, a fully virulent strain, producing shiga toxins 1 and 2, was fed to crickets *via* supplied drinking water for three days. All of the crickets survived until we disposed them 7 days after they were transferred to a new sterile cage. These results imply that the toxins produced by this pathogen are harmless to crickets. Another avirulent strain, not producing toxins, was fed to crickets also *via*

drinking water for 3 days. Out of the 20 test crickets, only 1 cricket, dissected on day 4, showed the presence of O157 in the hindgut. In the study, the test crickets were provided with a fresh O157 suspension at $9x10^8$/ml every day for 3 days. Assuming that the length of exposure and the dose of inoculation allowed crickets to ingest sufficient bacteria, O157 was unable to multiply and colonize in the hindgut of 95% of the test crickets. Either this pathogen could not compete with other microbial flora, or it could be killed by digestive enzymes or by the cricket's innate defensive mechanisms. Previous studies demonstrated O157 was not a member of normal flora in cricket's hindgut (Smith and Gaeddert, 2002). However, it is noteworthy that one cricket, dissected in the end of the 4^{th} day, showed the presence of O157 in its hindgut. This demonstrates that this cricket was able to acquire and harbor O157 for at least 4 days in its hindgut. It was also possible for this cricket to transfer O157 into the environment via the feces. Although we cannot rule out completely the possibility for crickets to transmit O157, house crickets do not appear to be a big threat for carrying this pathogen.

Under microscopic observation, a large portion of bacteria was associated with the cuticular structure of cricket's hindgut. This included the human opportunist pathogens: *Citrobacter; Klebsiella*, and *Yersinis*. No other microorganisms, such as protozoans, fungi, were observed (Ulrich et al., 1981). Most of the bacteria in the hindgut remained unidentified, mainly because these microbes are not culturable on the artificial media that were used; most of the cultivatable are facultative anaerobic bacteria. For this reason, molecular techniques recently

have been developed to detect the structure of the bacterial community in cricket's hindgut (Jorge, 1998; Jorge ibid., 1998). Even using these techniques, approximately 60% of the hindgut bacteria could not be characterized to species, but only into broad groups (Jorge ibid., 1998). Although the micro biota in cricket's hindgut is not obligatory for survival and growth of these crickets, they were shown to enhance the insects' ability to utilize a portion of the dietary carbohydrate from a variety of food sources (Kaufman et al., 1989). A further study by the same group of scientists demonstrated that bacteria in cricket's gut induced the production of enzymes and /or changes in bacterial population levels in response to the dietary changes in a natural environment (Kaufman et al., 1991). The shift of the hindgut microbial structure in crickets with the different diets was also demonstrated using group-specific probes (Jorge, et al., 1998). In our study, we fed crickets with Purina chicken feed and thus the hindgut microbial structure was likely very different from crickets living in the field. Unlike *Serratia marcescens*, *Salmonella typhimurium*, *Bacillus atrophaeus* and *E.coli* O157:H7 did not survive in cricket's hindgut for more than 6 days after inoculation. While our experiments demonstrated that crickets may harbor and transmit *S. marcescens*, the role of crickets in spreading the other pathogens is equivocal. However, due to the omnivorous diet of crickets, the physiological condition in wild crickets' hindguts should be different from those in our experiments. Therefore, it is possible that these three species of bacteria might survive and be disseminated in nature.

Cockroaches are also omnivorous. Compared to crickets, the structure of their microbial community in the digestive tract is much more complicated. Bacteria, fungi and parasites of medical importance all were isolated from cockroaches in different environments (Fotedar and Banerjee, 1992). Furthermore, a number of cases implicated cockroaches in transmission of drug-resistant strains of these microorganisms in hospitals and residential areas. Strains of *Serratia marcescens* and *Salmonella typhimurium* were isolated from cockroaches (Oothuman et al., 1989). These two species were not normally present in house cricket's hindgut. In previous studies, cockroaches fed with *Pseudomonas aeruginosa*, *Salmonella paratyphi* B var Java, and *Salmonella typhimurium*, were able to harbor and to transmit these pathogens (Fotedar et al., 1993; Singh et al., 1995; Klowden and Greenberg, 1976).

House crickets are similar to cockroaches in gut morphology and feeding behaviors (Cruden and Markovetz, 1987). Both insects are frequently encountered around humans' living areas. From the standpoint of dissemination of human pathogens, house crickets seem less harmful than cockroaches. While they may be able to acquire and harbor some potential human pathogens, this study provides little evidence for relating human diseases to house crickets.

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Table 1. The presence of *Serratia marcescens* of fed house crickets,

Aceta domesticus,.

Hours after inoculation	**Presence of *Serratia marcescens***	
	Hindgut	**Feces**
16	-	+
24	-	-
32	+	+
40	+	+
48	+	+
56	+	+
64	-	+

Table1. The presence of *Serratia marcescen* in the hindguts of house crickets, *Aceta domesticus*, inoculated through diet. One hundred microliters of inoculum of *Serratia marcescens* were applied into two milliliters of distilled water to feed the crickets; +, red colonies were recovered on TSA; -, no red colonies were recovered on TSA.

Table 2. The test of injection dose for house cricket, *Acheta domesticus*.

Concentration (bacteria/ml)	10^7	10^6	10^5	10^4	10^3
Sample Size	8	7	5	5	3
Death toll	4	4	0	1	2
Red body	4	3	0	0	0
Recovering *S.marcescens*	1	3	2	0	0

Table 2. The test of injection dose for house crickets, *Acheta domesticus*. Crickets were injected with *Serratia marcescens* at 10^7,10^6,10^5,10^4 and 10^3 bacteria/ml via esophagus; Sample Size, the number of crickets injected with bacterial suspension; Death Toll, the number of crickets dies during the process; Recovering *S.marsescens*, red colonies recovered from the hindgut samples on TSA; Red Body, the number of crickets died and their entire bodies turned red during the process.

Table 3. The presence of *Serratia marcescens* of injected crickets, *Acheta domesticus*.

Hours after injection	Presence of *S.marcescens*	
	Hindgut	Feces
8	-	NC
16	+	NC
24	+	-
32	+	-
40	+	-
48	+	-
72	+	NC
96	+	+
120	+	+

Table 3. The presence of *Serratia marcescens* in the hindguts of house crickets, *Acheta domesticus* inoculated by injection. These crickets were injected of 730- 1100 bacteria per cricket; +, red colonies were recovered on TSA; -, no red colonies were recovered on TSA; NC, no crickets survived.

Table 4. The presence of colonies of *Bacillus atrophaeus*, *Serratia marcescens* *Salmonella typhimurium* and *E.coli* O157:H7 recovered from house crickets, *Acheta domesticus*.

Day	*B.atrophaeus*		*S.marcescens*		*S.typhimurium*		Day	*E.coli* O157:H7	
	C	T	C	T	C	T		C	T
0	-	+	-	+	-	+	0	-	-
3	-	-	-	+	-	-	1	-	-
6	-	+	-	+	-	+	2	-	-
9	-	-	-	+	-	-	3	-	-
12	-	-	-	+	-	-	4	-	+
15	NC	NC	-	+	NC	NC			
18	NC	NC	-	+	NC	NC			

Table 4. The presence of colonies in the hindguts of house crickets, *Acheta domesticus* ,inoculated with *Bacillus atrophaeus*, *Serratia marsescens*, *Salmonella enteritidis* and *E.coli* O157:H7, respectively, through diet; C, control crickets; T, test crickets; +, the presence of tested colonies on plates; -, no tested colonies on plates; NC, no crickets survived.

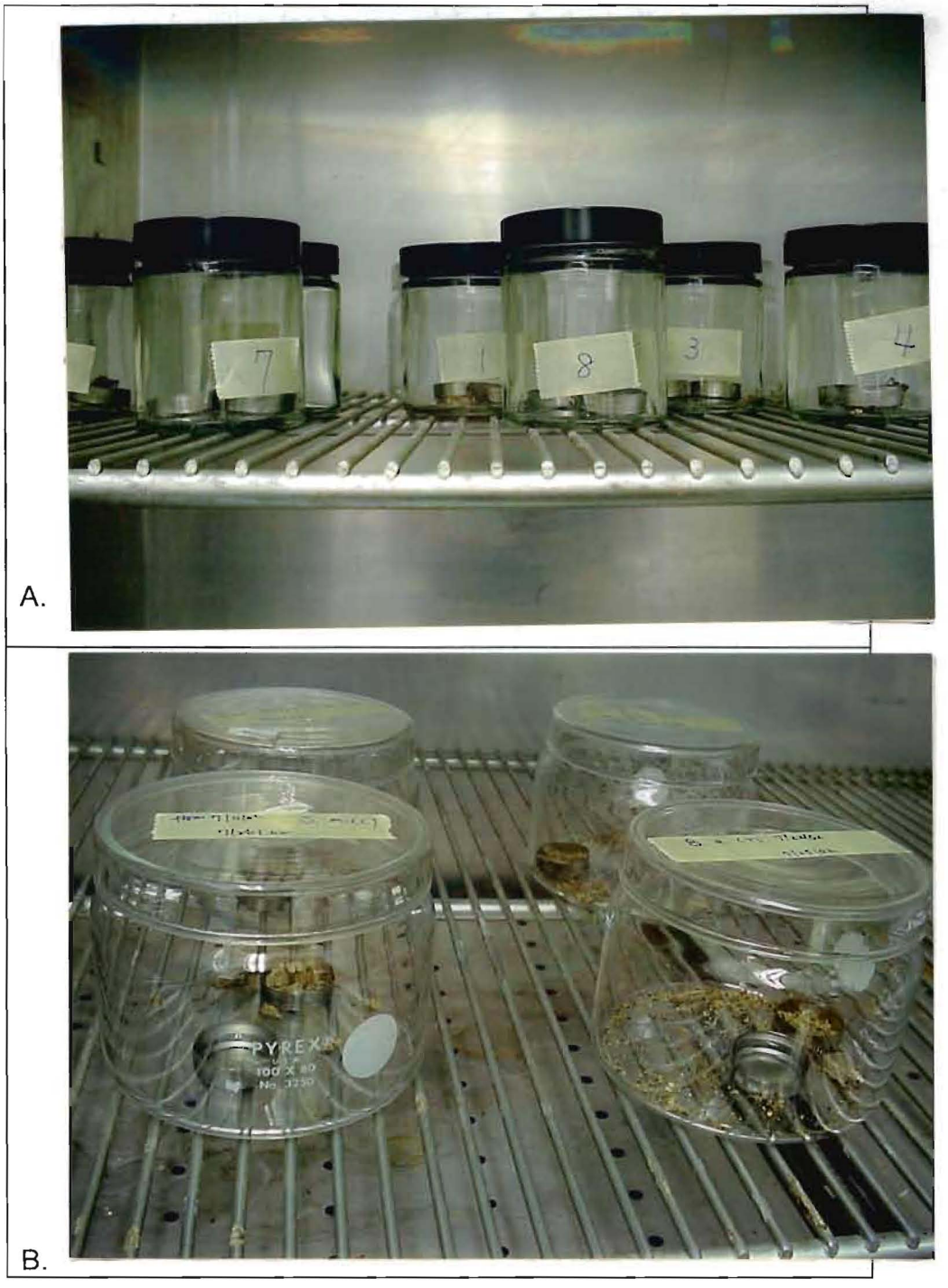
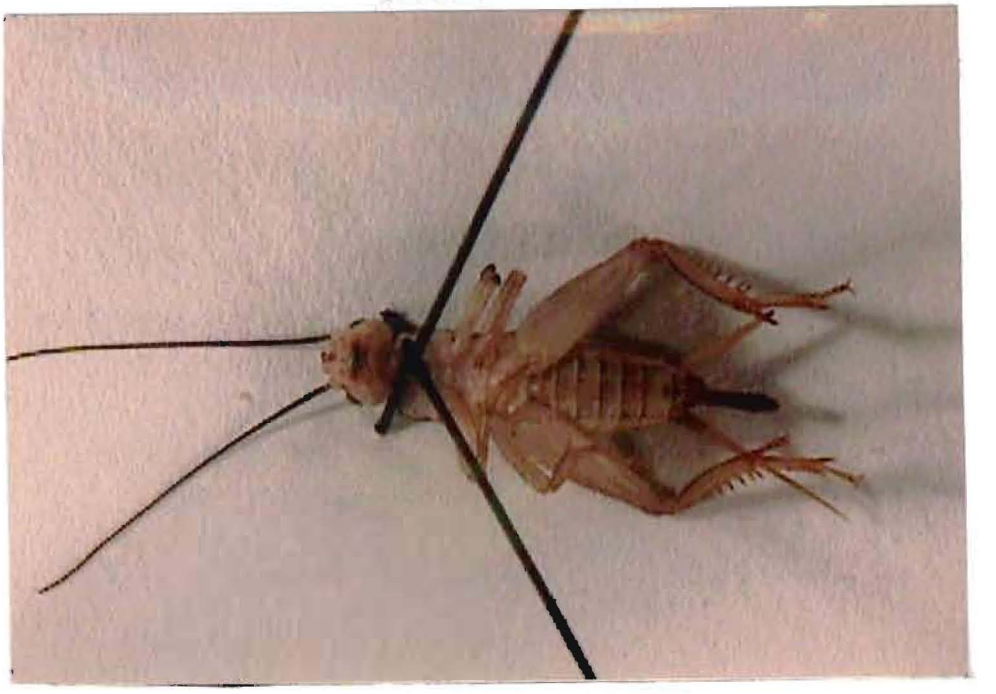


Fig. 1 The housing facilities for house crickets.

Fig. 1 The housing facilities for house crickets; A, the individual cages for house crickets in the pretests for testing the presence of a specific microbe; B, the cages for house crickets of harboring experiments.

A.



B.

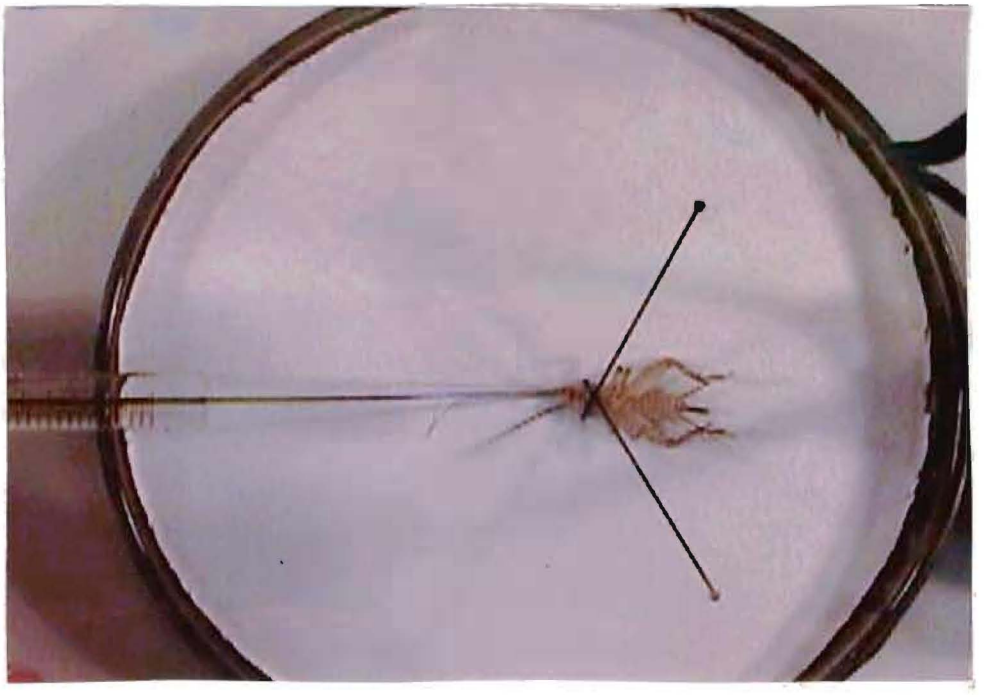


Fig. 2. Injection of House cricket, *Acheta domesticus.*

Fig. 2 Injection of house cricket *Acheta domesticus* with *Serratia marcescens;* A, the house cricket was set up to be injected with bacteria; B, the house cricket was injected with 10 µl of *S. marsescens* at 10^6 bacteria/ml.

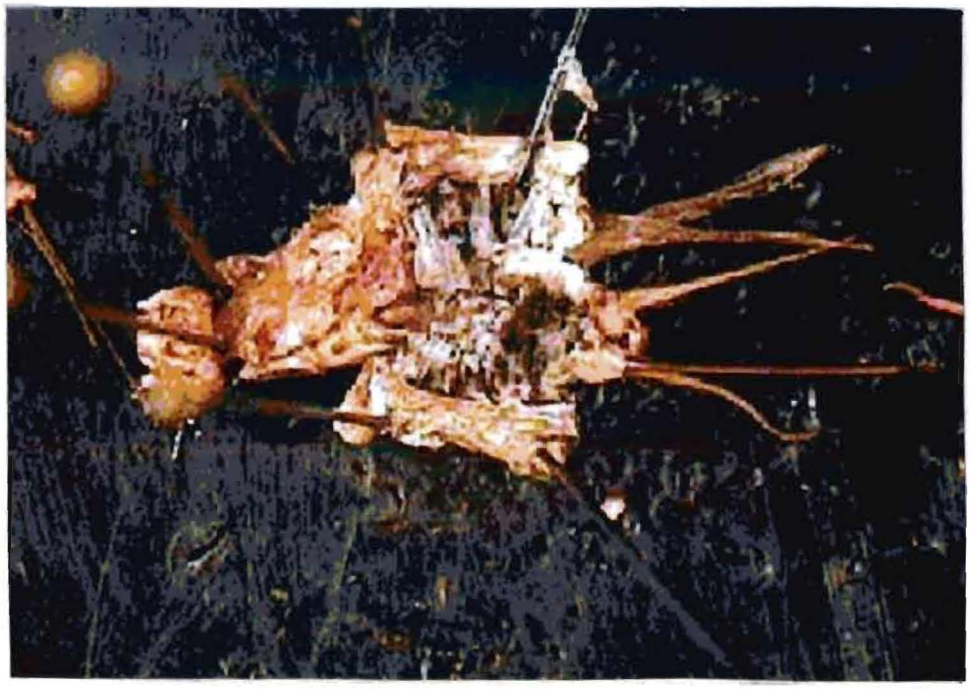


Fig. 3 The dissected house cricket, *Acheta domesticus*.

Fig. 3 The house cricket, *Acheta domesticus*, was dissected and opened its abdominal cavity.

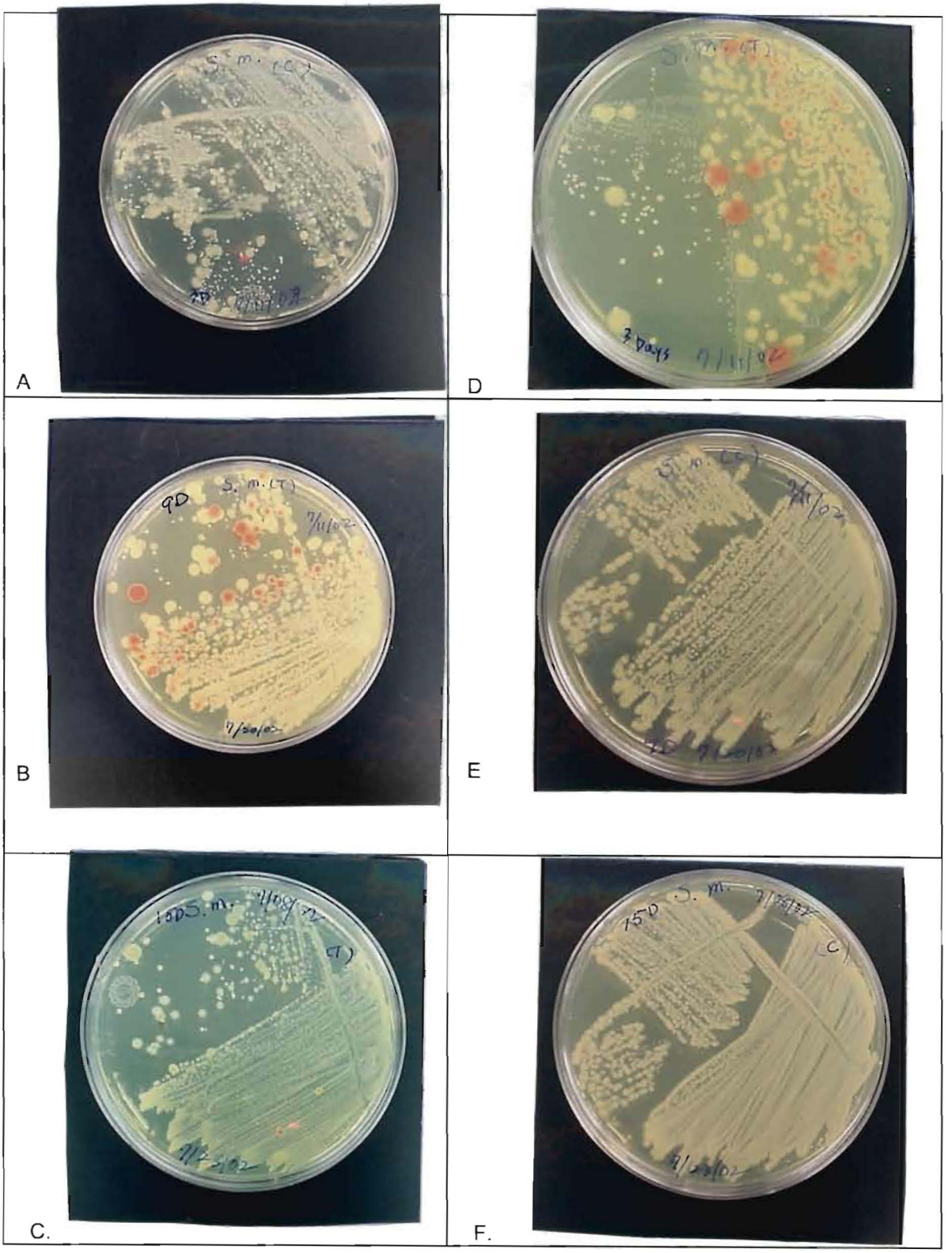


Fig. 4 The colonies of *Serratia marcescens* on the TSA plates.

Fig. 4 The colonies of *Serratia marcescens* were recovered from the hindgut samples from house crickets dissected on 0, 6, and 9-day post inoculation; A, from 0-day of test cricket; B, from 6-day of test cricket; C, from12-day of test cricket; D, from 0-day of control cricket; E, from 6-day of control cricket; F, from 12-day of control cricket.

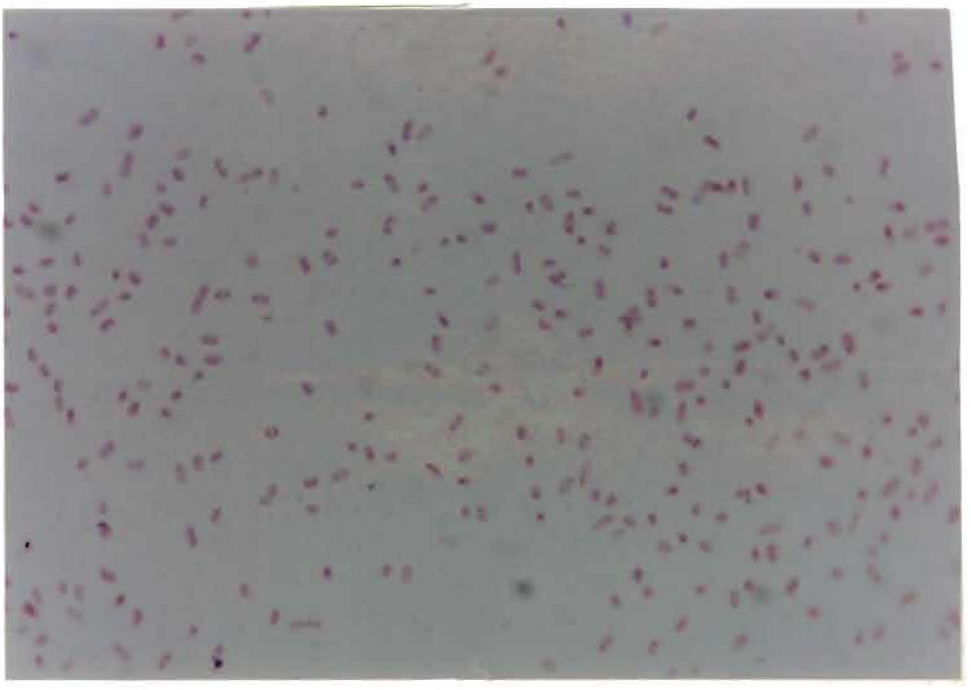


Fig. 5. Gram-stain of *Serratia marcescens.*

Fig. 5 Gram stain of *Serratia marcescens*. The colonies of *Serratia marcescens* subcultured from the red colonies on TSA which were recovered from the hindgut samples of test crickets.

A.

B.

C.

Fig. 6 The identification of *Salmonella typhimurium*.

Fig. 6 The identification of *Salmonella typhimurium*; A, *S. typhimurium* showed blue -green colonies with black centers on HE plates; B, the positive reaction of lysine decarboxylase test turned the broth deep purple; C, the indole test showed negative reaction.

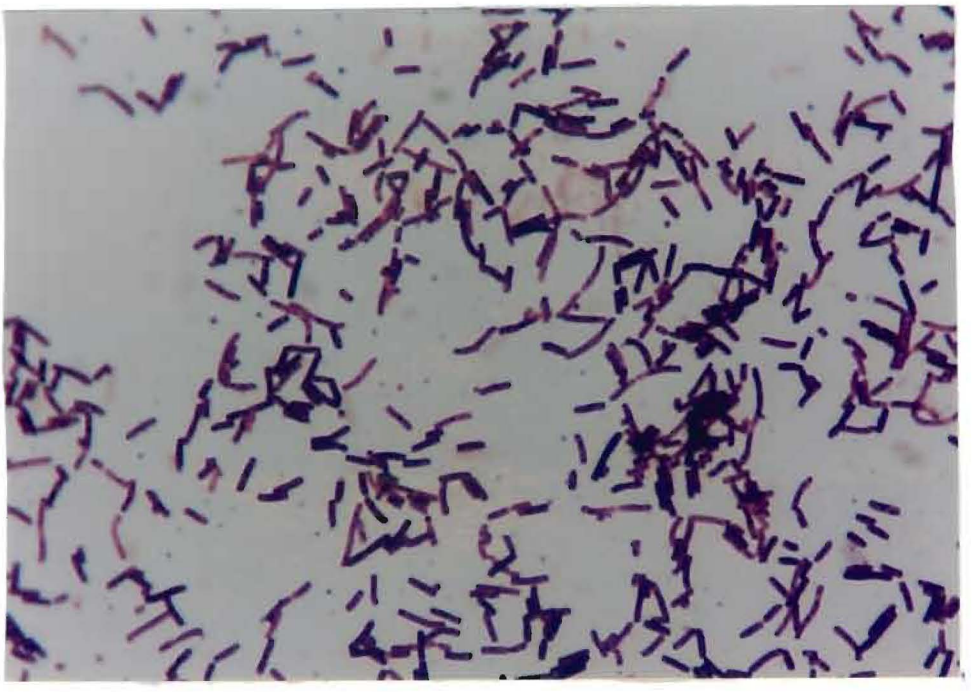


Fig. 7 Gram stain for *Bacillus atrophaeus* from pure culture on the TYE plate.

Fig. 7 Gram stain for *Bacillus atrophaeus* of recovered colonies from the hindgut samples of the test crickets. *B. atrophaeus* is a rod-shape, Gram-positive bacillus.

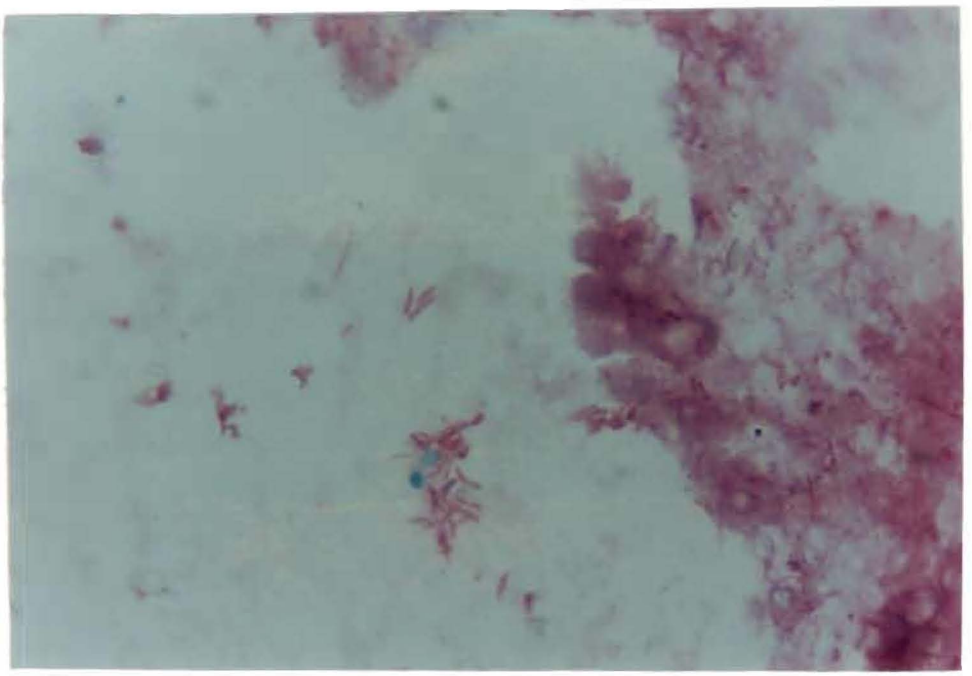


Fig. 8 Spore stain for the hindgut sample of 9-days cricket.

Fig. 8 Spore stain for the hindgut sample from the cricket of 9 days after inoculation of *Bacillus atrophaeus* spores mixed with feed. Note two bluish-green spores near center of lid.

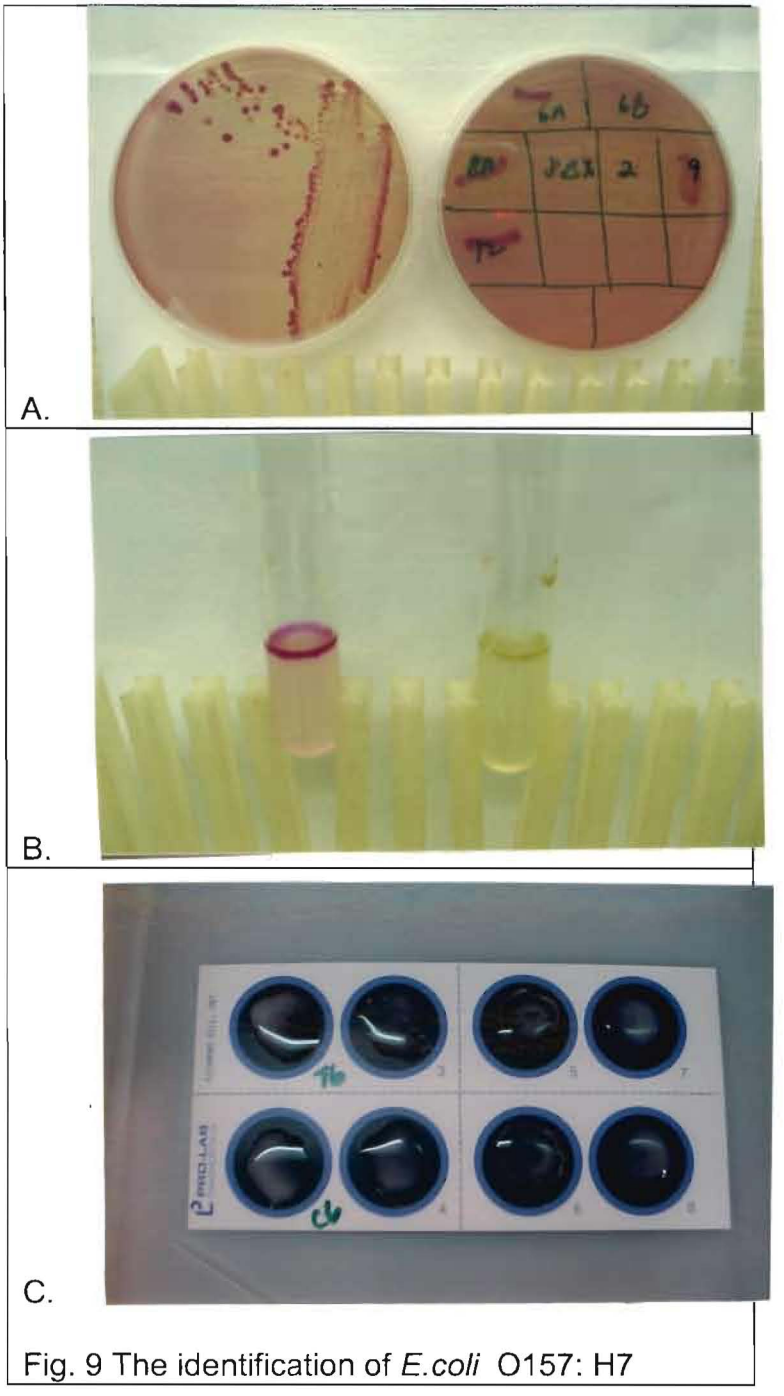


Fig. 9 The identification of *E.coli* O157: H7

Fig. 9 The identification of *E.coli* O157: H7; A, the colorless colonies of *E.coli* O157: H7 formed on the modified MacConkey plate; B, *E.coli* O157: H7 is indole –positive which showed a red ring on the surface of tryptophan broth; C, the serological test for *E.coli* O157 :H7, bacteria from the colonies of *E.coli* O157 :H7 agglutinated with the beads coated with anti-*E.coli* O157: H7.

Fig. 10 The house cricket, *Acheta domesticus*, was injected with and killed by *Serratia marcescens.*

Fig. 10 The house cricket, *Acheta domesticus,* died after being injected with *Serratia marcescens* and its entire body turned red (right).

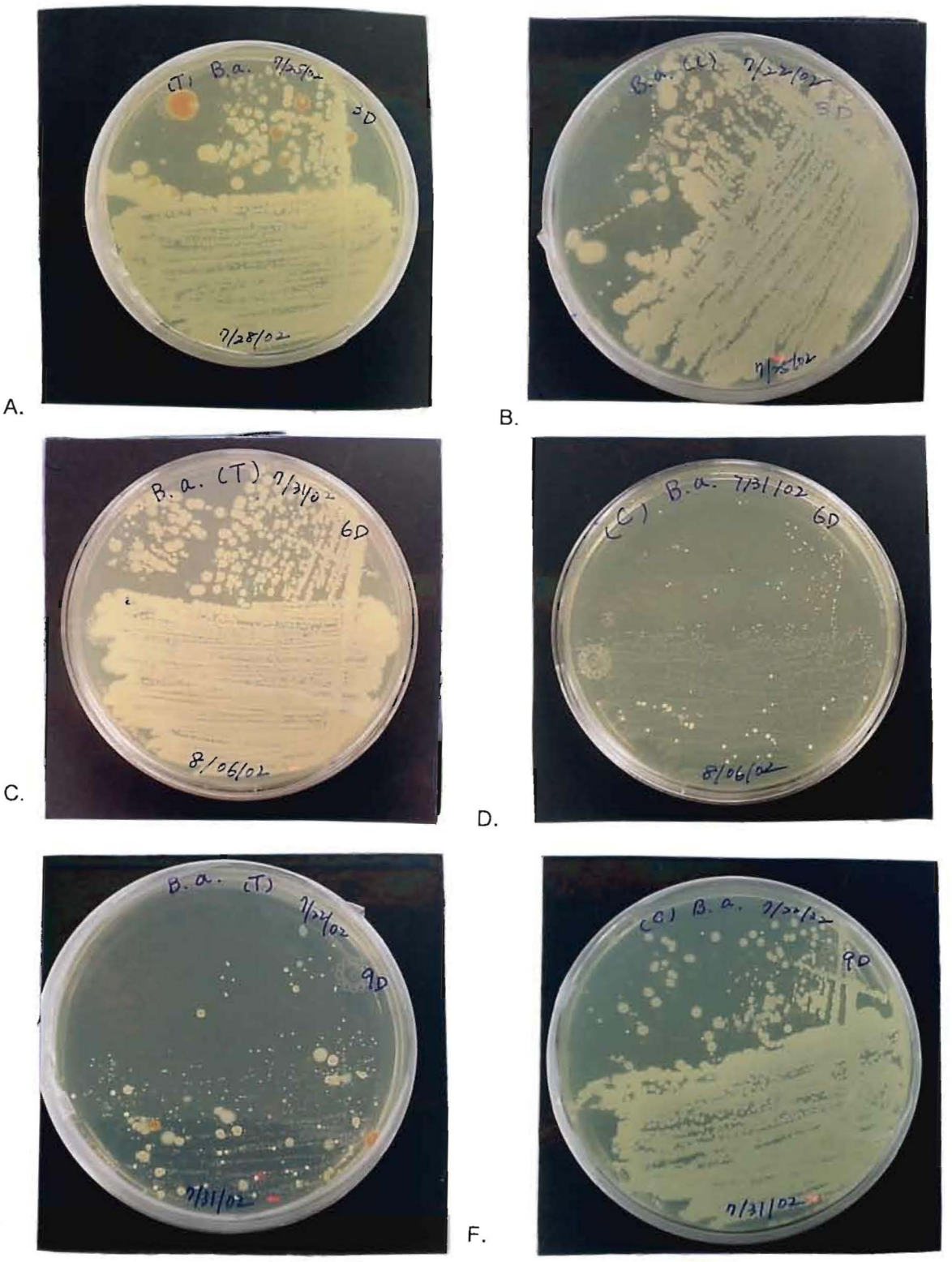


Fig. 11. The colonies of *Bacillus atrophaeus* on the TYE plates.

Fig. 11 The colonies of *Bacillus atrophaeus* were recovered from the hindgut samples of house crickets dissected on 0, 3, 6-day post inoculation: A, 0-day of test cricket; B, 0-day of control cricket; C, 3-day of test cricket; D, 3-day of control cricket; E, 6-day of test cricket; F, 6-days of control cricket..

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Tsai-Ying Liu

Signature of Author

Nov. 15, 2003

Date

The potential for house cricket, *Acheta domesticus*, to acquire, harbor, and transmit human pathogens

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