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Evaluating commercially available diagnostic tests for the detection of *Clavibacter michiganensis* subsp. *nebraskensis*, cause of Goss's bacterial wilt and leaf blight in corn

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by

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Evaluating commercially available diagnostic tests for the detection of *Clavibacter michiganensis* subsp. *nebraskensis*, cause of Goss's bacterial wilt and leaf blight in corn

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Goss's wilt and blight of corn, caused by the bacterium *Clavibacter michiganensis* (Cm) subsp. *nebraskensis* (Cmn), is currently diagnosed by symptom identification and successful isolation onto CNS selective medium. An ELISA test kit and ImmunoStrips (Agdia®) specific to Cm *michiganensis* (Cmm) reportedly give a cross-reaction with Cm subspecies. Also, the GEN III OmniLog Identification system (Biolog Inc., Hayward, CA) is said to provide accurate identification of Cm subspecies. These tests would provide a quick and inexpensive method for diagnosis of Cmn but have not been previously validated. ELISA test kits were provided by Agdia for the detection of Cmm, Cm *tessellarius* (Cmt), and Cm *sepedonicus* (Cms) as well as ImmunoStrips developed for the detection of Cmm. Also, an ELISA test kit (Neogen®) specific to Cmn was included in the study. For each ELISA and ImmunoStrip, 15 strains of Cmn, 5 Cmm, 5 Cmt, 4 Cms, 3 Cmi and 40 symptomatic leaf samples submitted from widely dispersed commercial corn fields to the UNL Plant & Pest Diagnostic Clinic were tested. The ImmunoStrips were tested with an additional 29 Cmn isolates as well as 17 bacterial isolates outside the genus *Clavibacter*. All ELISA test kits, except those for Cms, consistently gave positive results for all 40 infected leaf samples. Cmn was confirmed in the leaf samples with Koch's postulates. When testing cultures, the Cmn, Cmm, and Cmt ELISAs gave consistent positive results. The Cms ELISA gave consistent negative

results. ImmunoStrips for Cmm consistently tested positive for cultures of all the Cm subspecies and plant samples infected with Cmn and negative results for those bacteria outside the genus *Clavibacter*. ELISAs using antibodies specific to Cmm, Cmn and Cmt and ImmunoStrips using antibodies specific to Cmm can be used reliably when testing for the presence of Cmn. Further additions to the GEN III OmniLog ID system may be needed to provide consistent identifications of gram-positive coryneform phytopathogenic bacteria, namely those belonging to the genus *Clavibacter*

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DEDICATION

To my Friends and Family
For Their Unrelenting Support

Mom and Dad
Gina, Jenny, Jesse, Justin, Les

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Chapter I

Summary of Goss's Bacterial Wilt and Leaf Blight: An Economically Significant Disease of Corn

1.1 INTRODUCTION

According to the United States Department of Agriculture (USDA) and the National Agricultural Statistics Service (NASS), in 2009 the United States produced 13.2 billion bushels of corn (*Zea mays* L.) with an average of 165.2 bushels per acre. This is the largest amount of corn produced in one year to date. The production value of field corn in 2009 was estimated at $48.6 billion and sweet corn production reached $836 million. The threat that Goss's bacterial wilt and leaf blight poses to U.S. corn production is greater today than it ever has been in the past. In Nebraska alone, Goss's wilt has been reported in 62 counties since the most recent epidemic and is likely to exist in more counties than what has been officially reported (Tamra Jackson, personal communication, November 2010). The disease is residue-borne and once introduced to a field, the probability of it becoming a resident disease is relatively high (Schuster, 1975). Given the severity of Goss's wilt, and its rapid and unchecked movement throughout the Corn Belt, the need for the development and validation of diagnostic tests to accurately and rapidly identify the disease is highly warranted.

1.2 HISTORY

1.2.1 Distribution

In late August of 1969, in a field in Dawson Co., NE near the town of Lexington, a new foliar leaf blight of corn was observed. The blight symptoms were distinct yet the lesions resembled those induced by the known bacterial corn pathogen *Pantoea stewartii*

(formerly known as: *Erwinia stewartii*). Isolation of the casual agent via plant extractions however, revealed an orange pigmented, gram-positive bacterium that later became classified as a new species: *Corynebacterium nebraskense* (Vidaver and Mandel, 1974). The new disease was tentatively named Nebraska leaf freckles and wilt but was later changed to Goss's bacterial leaf blight and wilt after the former dean of the Graduate College and Chairman of the Department of Plant Pathology at the University of Nebraska – Lincoln, R.W. Goss (Schuster, 1970; Wysong et al., 1973).

By harvest of the following year, Goss's wilt was observed in nine different counties throughout Nebraska: Antelope, Dawson, Fillmore, Franklin, Furnas, Gosper, Nemaha, Platte, and York. The disease was confirmed in 72 fields throughout Nebraska and in over 100 different inbred lines from 17 different seed corn companies (Wysong et al., 1973). In 1971 the disease was identified in 11 new counties in Nebraska and Harrison County in western Iowa. The first publication of Nebraska leaf freckles and wilt on June 17, 1971 came in the form of an extension newsletter attempting to educate county agents on the signs and symptoms of the new disease (Wysong et al., 1971). Disease spread in 1972 was not unlike that of the previous year. Goss's wilt was found in three new counties in Nebraska and Norton County in northern Kansas.

Throughout the 1970's and 1980's the disease was confined to eight Midwestern states; Colorado, Illinois, Iowa, Kansas, Minnesota, Nebraska, South Dakota and Wisconsin (Wysong and Doupnik, 1981; Wysong et al., 1982; Wysong and Doupnik, 1984) After about 20 years of reduced disease incidence, Goss's bacterial wilt and blight re-emerged. Since 2006 the distribution of the disease has expanded to a total of 10 states in the U.S. (Colorado, Illinois, Indiana, Iowa, Kansas, Minnesota, Nebraska, South

Dakota, Texas, Wyoming), and two providences in Canada, Manitoba (Vikram Bisht, personal communication) and Ontario (Ruhl et al., 2009; Wysong et al., 1983).

1.2.2 Causal Agent

Goss's wilt is caused by a bacterium originally classified as *Corynebacterium nebraskense* (Vidaver and Mandel, 1974). Later it was placed in the genus *Clavibacter*, of the family *Microbacteriaceae* (Park et al., 1993). The genus *Clavibacter* contains only one species *C. michiganensis* (Cm). *C. michiganensis* consists of five subspecies: *C. michiganensis* subsp. *michiganenses* (Cmm), *C. michiganensis* subsp. *nebraskensis* (Cmn), *C. michiganensis* subsp. *insidiosus* (Cmi), *C. michiganensis* subsp. *tessellarius* (Cmt), and *C. michiganensis* subsp. *sepedonicus* (Cms) (Carlson and Vidaver, 1982; Davis et al., 1984; Riley and Ophel, 1992;). Each of the five subspecies of *C. michiganensis* is a host specific plant pathogen.

This morphologically distinct group of bacteria is characterized as Gram positive, rod or coryneform shaped, and non-motile. Average cell dimensions of Cmn grown at 24 C for 40 h are approximately 0.5 by 2.0 µm (Schuster, 1975; Vidaver and Mandel, 1974).

1.3 IMPACT ON CORN PRODUCTION

1.3.1 Occurrence of Pathogen within the Host and Symptom Expression

Goss's bacterial leaf blight and wilt (as it implies) is characterized by two distinct phases. The wilt systemic phase, associated with early infection, results from the release of extracellular polysaccharides (EPS) by the bacteria that inhabit the xylem of infected plants. The gummy or slimy to mucoid EPS produced by the bacteria blocks the water conducting elements within the plant, resulting in wilt symptoms and oftentimes mortality before the plant is able to reach maturity (Schuster, 1975).

The second, the leaf blight phase, is the most common and produces the most diagnostic symptoms. The bacterium produces a phytotoxin that is released ahead of the bacteria and helps destroy plant tissue. As infection progresses, long wavy lesions develop and are often accompanied by irregular, dark, discontinuous water-soaked spots that occur on the margins of the lesions just ahead of the necrotic tissue and are often referred to as freckles. Also, a glossy exudate may be secreted onto either the top or bottom leaf surface or both. Cmn has been reported to cause infection in every part of the corn plant. Each plant part infected produces a distinct yet similar symptom indicative of Cmn infection (Schuster, 1975). Wounding is believed to be necessary for infection to occur and can come in several forms; insect feeding, leaf tatter from wind, hail damage and abrasion by blowing sand (Rocheford, et al., 1985). The leaf blight symptoms are similar in appearance to those produced by Stewart's wilt. Resistance is highly correlated between the two diseases as marked by relatively high rank correlation coefficients. Therefore, in some screening trials, hybrid lines with susceptibility and resistance to Stewart's wilt were selected for evaluation of their relative resistance and susceptibility to Goss's wilt (Carlson and Wicks, 1991; Pataky, 1985). However, Stewart's wilt can be differentiated visually from Goss's wilt by the absence of discontinuous water soaked spots and the lack of a glossy sheen.

Stems may become infected if the bacterium is able to enter directly or if infection occurs at an early growth stage and becomes a systemic infection (Eichenlaub et al., 2006). Stem infections are not always apparent and a cross-section of an infected stem will reveal the orange to brown exudate occupying the vascular elements. Once the bacterium has entered the vascular tissues of the corn plant, depending on the amount of

time available, aggressiveness of the strain and the susceptibility of the host – it may spread to the shank, cob and even into the kernels. In these cases, infection is always identified by the orange to brown exudate produced as an EPS by Cmn (Schuster, 1975).

Cmn is able to occupy the internal elements of corn seeds and maintain viable population on the external parts of the seed. Infection was observed by Schuster (1975) when the cross-section of a kernel revealed pockets of orange ooze adjacent to the chalazal region. Inoculation of these exudates into susceptible corn varieties showed that the kernel can indeed become infected. The transmission rate of Goss's wilt from seeds to seedlings is very low. A greenhouse study revealed that only 0.8% of naturally infected seed that germinated resulted in the subsequent infection of the seedling (Schuster, 1975). A similar study by Biddle et al., 1990, revealed comparable average transmission rates of 0.1 to 0.4%. However, in this study, infested seed used was the result of artificially inoculated seed corn and no transmission was observed from naturally infected seed.

Infection of Cmn through the roots was also observed both when corn plants were potted in soil in the greenhouse and when grown in hydroponics. In both circumstances, root wounding was necessary for infection to occur (Schuster, 1975). Mortality and incidence of infection were both increased in alfalfa (*Medicago stiva* L.) when inoculated with *C. michiganensis* subsp. *insidiosus* in the presence of a root-knot nematode (*Meloidogyne hapla* Chitwood). Root wounding by sedentary nematodes allows for the entry and subsequent exacerbation of wilt symptoms of alfalfa caused by the wilt pathogen Cmi (Hunt et al., 1970). This study suggests that Cmn bacteria may have the capability of increasing disease incidence and severity if in direct association with soil

organisms that occupy the same ecological niche and produce wounds on the roots of corn.

The effect of plant age and inoculum concentration was explored in a study by Calub et al, (1974). The dependence of plant age on disease spread varied between susceptible and resistant lines. There was little difference observed in disease severity according to age when susceptible lines were inoculated at different growth stages. However, it was noted that the most severe disease ratings occurred when susceptible lines were inoculated after eight weeks of growth, at which time the corn plant was tasseling. An increase in age, for the resistant lines tested, subsequently resulted in a decrease in disease severity. As expected, regardless of the hybrid or inbred being tested, there was an increase in disease severity as the concentration of inoculum increased. When plants were inoculated with bacteria at a concentration of 10^2 cells/ml no observable disease was recorded. When plants were inoculated with bacterial concentrations of 2×10^8 cells/ml advanced Goss's leaf blight symptoms developed, though symptom severity decreased with increasing plant age.

Mechanisms involved in movement of Cmn within the cornplant have not been fully elucidated. However, studies with Cmm and Cmi have revealed some insight as to the method of pathogen movement within the host plant as well as some of the key bacterial enzymes involved. The downward movement of Cmm in inoculated tomato (*Lycopersicon esculentum* L.) plants was detected 10 cm below the initial point of infection 7 days after inoculation (Gitatis et al., 1991). Entry into the phloem of host plants by Cmm and Cmi is facilitated by cell wall degrading enzymes such as cellulose,

polygalacturonase, and xylanase (Marte, 1980; Benhamou, 1991; Baer and Gudmestad, 1995; Beimen et al., 1992).

1.3.2 Pathogen Persistence and Dispersal

As stated previously, Cmn is a residue borne pathogen, as such, practices like continuous corn cropping and reduced tillage systems allow for the perpetuation and long term persistence of the pathogen in corn fields (Schuster, 1975, Schuster and Coyne, 1974). It has been suggested that increased inoculum load from subsequent years of disease incidence can significantly increase disease severity for the following year (Pataky et al., 1988.)

1.3.3 Impact on Yield

The terms "susceptible" and "resistant" are merely the endpoints of a continuum scale of reactions that will occur when a corn plant is inoculated with Cmn. There is not a single hybrid or inbred that is completely resistant to Cmn. Host reactions are subject to a myriad of factors such as environmental conditions, inoculum concentration, and plant age at the time of infection.

It was discussed in section 1.3.1 that the disease's impact on yield can be directly related to its severity. The results of earlier research indicate that yield reduction is more severe when plants are infected at an earlier growth stage and symptom expression is mediated by inoculum concentration (Suparyono and Pataky, 1989; Claub et al., 1974). A maximum yield loss of 43.5% was recorded in a controlled inoculation study of A632-type inbred lines with varying levels of resistance. A significant correlation was made between disease severity and yield loss suggesting that disease ratings may be an adequate indication of the relative resistance of a given inbred or hybrid. For example, a

disease rating of 2 (slight wilting around pinprick wounds) or less correlated with insubstantial yield loss which suggests that significant movement of symptoms from the point of infection must occur before significant yield loss is recorded. However, some inbreds in the study with disease ratings that were not significantly different produced yields that were significantly different. This interaction implies that some inbreds may be more tolerant of the disease than others. However, discrepancies in the data between identical treatments of tests conducted during different years, suggests that tolerance could be effected by environmental conditions including the age of the plant at the time of the pathogen introduction (Carson and Wicks, 1991). Because disease severity is only measured by the amount of visible damage, it is possible that the true severity is unknown; especially considering that Cmn is a vascular pathogen that can disrupt the water conducting elements of the host plant.

In most inoculation studies a bacterial concentration of 10^7 CFU/ml was most effective for the production of symptoms regardless of the plant's growth stage (Calub et al., 1974; Pataky et al., 1988; Saparyono and Pataky, 1989). Plant age had a small effect on disease severity ratings for susceptible hybrid lines but played an important role for resistant lines. In general disease severity was reduced when plants were inoculated closer to plant maturity (Calub et al., 1974; Saparyono and Pataky, 1989).

Sweet corn yields – as measured by kernel weight, number of marketable primary ears, total number of ears and ear diameter – were affected the most when susceptible and moderately susceptible varieties were inoculated at the 3-5 leaf stage. Total husked ear weight was reduced by as much as 50% when a moderately resistant variety was inoculated and 99% when a susceptible hybrid was inoculated. All percentages were

based on non-inoculated control plots. Similarly the number of marketable primary ears was reduced by 95% when a susceptible hyrbrid was inoculated at the 3-5 leaf stage. Reductions in ear diameter and length resembled those of the other yield determinants, with the most severe reductions occurring on the susceptible and moderately susceptible varieties inoculated at the 3-5 leaf stage. A considerably lower yet still significant yield loss was recorded when susceptible varieties were inoculated at the 5-7 leaf stage (Pataky et al., 1988; Saparyono and Pataky, 1989).

1.4 *CLAVIBACTER MICHIGANENSIS* SUBSPECIES

1.4.1 Classification and Characterization

Clavibacter michiganensis subsp. *michiganensis* was first identified as the cause of a wilt of tomato (*Lycopersicon esculentum* Mill.) in 1909 near Grand Rapids Michigan. Isolation from infected tomato plants revealed a gram positive club or rod shaped bacterium that formed colonies that were approximately 10µm in diameter. The colonies were canary yellow, smooth, shiny, opaque, relatively flat and viscid. The bacterium was originally classified as *Bacterium michiganense* (Shear, 1910). In 1925, another gram positive club-shaped bacterium was isolated from alfalfa (*Medicago sativa* L.) plants that were displaying wilt symptoms. Colony morphology was similar to that of *Bacterium michiganense* the convex colonies were flat and viscid, smooth, shiny, circular with entire margins and white to pale yellow. The bacterium was classified as *Aplantobacter insidiosum* (McCulloch, 1925). A review of the classification schemes of the genera *Mycobacteria* and *Corynebacteria* resulted in the reclassification of both of the afore mentioned bacteria as *Corynebacterium michiganensis* and *Corynebacterium insidiosum*, respectively (Jensen, 1934).

Clavibacter michiganensis subsp. *sepedonicus* was first isolated from potato (*Solanum tuberosum* L.) in 1913 and classified as *Bacterium sepedonicum*. However, insufficient character descriptions in the first publication and poor author citations in subsequent publications resulted in confusion among investigators (Smith, 1920). The bacterium was placed into two different genera (*Aplantobacter* spp. and *Phytomonas* spp.) throughout the 1920's and 1930's (Smith, 1920; Savile and Racicot, 1937). Jensen, 1934 suggested that the bacterium be placed in the genus *Corynebacterium*, based on its physiological and morphological characteristics (gram positive pleomorphic rods, non-motile displaying "snapping division"). However, the bacterium was not officially placed into the genus *Corynebacterium* until 1942 (Skaptason and Burkholder, 1942).

In 1976 a gram positive club-shaped bacterium was discovered as the cause of a foliar mosaic disease of wheat (*Triticum aestivum*). Water-soaking and bacterial streaming were not present, however, the cultural characteristics of the bacteria were similar to other corynebacteria (convex, glistening with entire margins, circular 2-4 mm in diameter, butyrous, and apricot orange - though pigmentation varied with media type). The bacterium was called *Corynebacterium michiganense* subsp. *tessellarius* (Carlson and Vidaver, 1982).

The four species of *Corynebacterium* (*C. iranicum*, *C. tritici*, *C. rathayi*, and *C. michiganense* were re-classified and placed into the genus *Clavibacter*. Furthermore, *Corynebacterium michiganense* subsp. *michiganense* became *Clavibacter michiganensis* subspecies *michiganensis*, *Corynebacterium michiganense* subsp. *insidiosum* became *Clavibacter michiganensis* subspecies *insidiosus*, *Corynebacterium michiganense* subsp. *sepedonicum* became *Clavibacter michiganensis* subspecies *sepedonicus*,

Corynebacterium michiganense subsp. *tessellarius* became *Clavibacter michiganensis* subspecies *tessellarius*, and *Corynebacterium michiganense* subsp. *nebraskense* became *Clavibacter michiganensis* subspecies *nebraskensis*. These four species are classified as such according to the presence of 2, 4-diaminobutyric acid in the peptidoglycan layer of the cell wall (Davis et al., 1984). Please refer to table 1 for a list of the five Cm subspecies and the diseases caused on their respective plant hosts.

1.5 *CLAVIBACTER MICHIGANENSIS* SUBSPECIES *NEBRASKENSIS*

1.5.1 Biology

Cultural differences can be observed between type strains of Cmn or when the same strain is plated on different forms of media or the same media at different temperatures. The most common characteristics include a round, dark-orange or apricot-orange colored colony. Colonies are mucoid, and the center has a darker hue than the margins which are entire. Colonies are usually shiny with a convex shape. The diameter of most colonies after 5-7 days of growth at room temperature is 3-5 mm (Vidaver and Mandel, 1974). Most colonies are smooth in character but some strains produce rough colonies. Both colony types can be equally virulent. Attenuation of isolates was observed after continued *in vitro* propagation and was not restored even after being placed in susceptible host plant tissue five successive times. Inoculum additives that often aid in the restoration of virulence (cyclic AMP, amino acids or nitrogen forms) had no effect on attenuated Cmn isolates (Schuster, 1975).

1.5.2 Growth Response to Temperature

The optimal growth range for Cmn is between 24 to 28 C. The shortest doubling times, both in planta and when grown in NBY broth, were recorded at 26 C and were 3.9

and 3.5 h, respectively. When propagated on NBY agar or in NBY broth the thermal death point was reached at 37 C. Growth was maintained but retarded at 10-12 C (Smidt and Vidaver, 1986; Vidaver and Mandel, 1974; Schuster, 1975).

1.5.3 DNA Analysis

DNA analysis of six Cmn isolates revealed a 73.5% guanine + cytosine ratio (Schuster, 1975). The majority of plasmids found in gram-positive, coryneform phytopathogenic bacteria, though differing in size and number, consist mostly of covalently closed circular (CCC) DNA. It was discovered after cell lysis and isolation of plasmid DNA that 5 out of 22 Cmn isolates contained CCC plasmids, and those that did carried a low copy number, usually only 1 to 2 copies per cell. In Cmn there is no correlation between the presence of plasmids and the production of bacteriocin. Furthermore, strains containing the same number of plasmids of the same relative molecular weight differed in basic morphological and physiological characteristics such as pigmentation, colony morphology, bacteriocin production and virulence (Gross et al., 1979). Cmn is capable of producing bacteriocin CN1 exclusively or bacteriocins CN1 and CN2 together, both strains with and without plasmids were capable of producing these toxins. Gross and Vidaver, (1978) tested 52 strains of Cmn for their capability to produce bacteriocin, all 52 strains were capable of producing CN1 and 19 of these strains produced CN2. Both CN1 and CN2 are bactericidal proteins that inhibit the growth of other bacteria, even within the same species. Most strains not capable of producing CN2 were inhibited by CN2, and all of the strains capable of producing the protein were resistant.

There are bacteriophages specific to Cmn that belong (as do all phages specific to corynebaceria) to styroviridae. They have "isometric heads and long flexuous, non-contractile tails. Research conducted on phages specific to Cmn has focused on the characterization of the physical and biological properties of the phages and not on the parasitism or interaction with the bacteria (Shipako et al., 1986).

1.6 HOST SPECIFICITY OF *CLAVIBACTER MICHIGANENSIS*

Each of the five subspecies of Cm is specific to its respective hosts (Table 3). Cmm causes a wilt of tomato. Cmn causes a wilt and leaf blight of corn. Cmi causes wilt of alfalfa. Cmt is responsible for a bacterial mosaic in wheat and Cms will cause a bacterial ring rot of potato (Eichenlaub et al., 2006).

1.6.1 Alternate Hosts of Cmn

The primary host of Cmn is *Zea mays*, though disease severity may fluctuate between the various hybrids and inbreds (Calub et al., 1974a; Calub et al., 1974b; Carlson and Wicks; 1991; Martin et al., 1975; Ngong-Nassah et al., 1992; Pataky, 1985; Pataky et al., 1988; Suparyono and Pataky, 1989; Schuster et al., 1972). Natural infections of Cmn were observed in green foxtail (*Setaria viridis* L.), shattercane (*Sorghum bicolor*), field and sweet corn. Greenhouse inoculations with Cmn produced similar symptoms on teosinte (*Euchlaena mexicana*), Eastern gama grass (*Tripsacum dactyloides*), and green foxtail. Greenhouse inoculation of Cmn produced dissimilar symptoms on shattercane, grain sorghum (*Sorhum vulgare*), sudangrass (*Sorhum vulgare sudanse*) and sugarcane (*Saccharum officinarum*). Irregular symptoms included red veins and long red or small irregularly shaped lesions that ran parallel to the veins. Discontinuous water-soaked spots did not appear in association with these lesions (Schuster, 1975).

1.7 OVERWINTERING CAPABILITIES

The bacterium overwinters in plant residue on the soil surface and less successfully in soil. Burying residue is an effective way to reduce inoculum load. It was also observed that survival of Cmn was higher when infected leaves were air dried before being buried and lower when moist leaves were buried. This study involved burying infected leaf tissue, stems, cobs, ears and pure cultures of the bacterium. Survival at three soil depths was compared: 0, 4, and 8 inches. Survivability of the pathogen was measured by recovering the tissues, soaking them in water for 4 hours and then using the supernatant to inoculate susceptible corn plants (Schuster, 1975).

1.8 ISOLATION

Some of the most prevelant and persistant corn endophytes and epiphytes are gram positive coryneforms that are closely related to Cmn. These endophytes belong to the genera *Cellulomonas*, *Curtobacterium* and *Microbacterium* (Zinniel et al., 2002). Because the growth and nutrient requirements of closely related bacteria are often similar, a selective medium was needed for the isolation Cmn.

In 1979 Gross and Vidaver developed a semi-selective medium for the isolation of Cmn. *Corynebacterium nebraskense* selective (CNS) media is useful for both the qualitative and quantitative isolation of Cmn. This medium allows for the isolation of Cmn from either fresh corn plant tissue, dry residue, or soil which is helpful in monitoring inoculum levels across a field or larger geographic area. CNS is considered only semi-selective because of the ability of other gram positive, coryneform bacteria to grow on the medium. These include bacteria in the genera *Corynebacterium* and *Arthrobacter*. Of the genus *Corynebacterium*, several species grow well on CNS,

including *C. nebraskense* and *C. michiganense* (now *Clavibacter michiganensis* subsp. *nebraskensis* and *Clavibacter michiganensis* subsp. *michiganensis,* respectively). *Corynebacterium tritici*, *Curtobacterium flaccumfaciens* pv. *betae* (syn. *Corynebacterium betae*) and *Curtobacterium flaccumfaciens* pv. *oortii* (syn. *Corynebacterium oortii*) were also among the species to grow with relative success on CNS media. Conversely, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (syn. *Corynebacterium flaccumfaciens*), *Curtobacterium flaccumfaciens* subsp. *poinsettiae* (syn. *Corynebacterium poinsettiae*), and *C. rathayi* displayed poor plating efficiencies while *Clavibacter michiganensis* subsp. *insidiosus* and *C. michiganenses* subsp. *sepedonicus*, *Corynebacterium iranicum* and *Corynebacterium fascians* were inhibited completely (Collins and Jones, 1983; Gross and Vidaver, 1979).

The active inhibitory ingredients of CNS medium consist of naladixic acid a bacterial antibiotic, lithium chloride (which is often omitted because of its toxicity to freshly transferred Cmn colonies) and Bravo 6F, a fungicide with the active ingredient chlorothalonil (Gross and Vidaver, 1979).

1.8.1 Maintenance and Viability of Cultures

Freeze dried lyopholization is the best means of long term storage of Cmn to maintain both viability and virulence (Vidaver, 1976). Virulence was maintained for up to 2 years when strains were plated onto $CaCO_3$ medium and then stored at 6 C. Strains plated on NBY and then stored at 6 C for 2 years were less virulent. Strains plated onto SSM (semi-synthetic medium) and then subsequently stored at 6 C for 2 years displayed very poor virulence. Distilled water kept at room temperature proved to be unsuitable to maintain either virulence or viable Cmn cell cultures.

1.9 DIAGNOSTIC PROCEDURES

CNS selective medium has been developed for the isolation of Cmn, however, as discussed in section 1.8, solely relying on its selectivity can be risky considering the array of gram-positive coryneform bacteria that are able to grow on the medium. If isolations are made on non-selective media, Gram staining is a useful tool for differentiating between gram-positive and gram-negative bacteria. The similarity among symptoms produced by Goss's wilt and Stewart's wilt make diagnosis difficult to the untrained eye. A simple Gram stain will reveal the casual agent as Cmn is gram-positive and *Pantoea stewartii* is gram negative.

1.9.1 Serological Diagnostics

There are several serological diagnostic assays that exist to detect Cm subspecies. The enzyme linked immunosorbent assay (ELISA) and the lateral flow chromatograph are two examples of testes used to detect bacterial plant pathogens (de León et al., 2007; De Boer et al., 1994; Drennan et al., 1993; Gitatis et al., 1991; Gudmestad et al., 2009; Kaneshiro et al., 2006; Lee et al., 2001; Slack et al., 1996;). The problem that arises in pathogen detection using serological methods is the lack of specificity of the available antisera. Specific antigen detection is difficult in gram-positive bacteria because of the relative ubiquitous nature of the antigen determinants, namely teichoic acids peptidoglycans and capsular polysaccharides (Myerowitz et al., 1973; Schleifer and Seidl, 1977; and Wicken and Knox, 1975). Several studies have clearly revealed the ability of most antisera developed for the detection of a particular Cm subspecies to cross-react with another non-target subspecies of Cm (Alvarez et al., 1993; De Boer, 1982; De Boer and Copeman, 1980; Franken et al., 1993; De Boer et al., 1988; De Boer

and Wieczorek, 1984). In some cases these antisera have been known to cross-react with other bacteria outside the genus *Clavibacter*, as well as other saprophytic, unidentified coryneform bacteria (Calzolari, et al., 1982; De Boer and Copeman, 1980; De Boer et al., 1988; De Boer and Wieczorek, 1984; Slack et al., 1979). Occasionally, antisera developed for the detection of a gram-positive bacterium will react with a gram-negative bacterium (De Borer et al., 1988). Some of the cross-reactivity of antibodies can be eliminated by developing monoclonal antibodies. (De Boer et al., 1988;). *C. michiganensis* subs. *sepedonicus,* though very closely related to the other subspecies of Cm, was at one time categorized separately due to differences in nucleoprotein and polysaccharide antigens (Schuster et al., 1975).

Although cross-reactions may occur in immunofluorescent microscopy, unrelated bacteria can be differentiated by differences in cell morphology (Franken et al., 1993). De Boer and McNaughton, 1986 used immunofluorescence to detect low population densities of Cms in potato stems and tubers (one cell per microscope field or 1.5×10^3 cells/g of potato tuber tissue). Low bacterial titers were also detected with a closely related indirect fluorescent antibody stain (IFAS) test. Populations as low as 10^1-10^2 cells/ml were detected (Slack et al., 1978). IFAS was even useful in detecting latent infections in symptomless tubers. Although this procedure is very sensitive to low bacterial populations, slide preparation is time consuming and laboratories are required to have expensive microscopes with the appropriate filters for fluorescein fluorescence with a viewing field of at least 1000x magnification (De Boer and McNaughton, 1986).

Latex agglutination has been shown to be effective for the detection of Cms at bacterial concentrations of 10^6 CFU/ml. However, cross-reactions to *Curtobacterium*

flaccumfaciens (syn *Corynebacterium flaccumfaciens*), *Curtobacterium flaccumfaciens* pv *poinsettiae* (syn *Corynebacterium poinsettiae*) and Cmm were observed (Slack et al., 1979).

1.9.2 Molecular Diagnostics

In an attempt to find reliable and accurate PCR primers to isolate and sequence the 16S to 23S rRNA region of the genome of *Clavibacter michiganensis* subsp. *sepedonicus*, primers were also found to isolate the same region of Cmn as well as the other 3 subspecies of Cm. Both the forward and reverse primers were found to be reliable for the amplification of the 16S to 23s rRNA region of all 5 subspecies of *Clavibacter michiganensis* as well as 3 specieces of *Rathaybacter*; *R. rathayi*, *R. iranicus,* and *R. tritici*. The primers also amplified the same region of the genome for *Erwinia carotovora* subsp. *astroseptica* (Li and De Boer, 1995).

Several techniques have been developed for the detection and differentiation of the 5 subspecies of Cm. In 1998, Louws et al. used a repetitive-sequence-based (rep)-PCR genomic fingerprinting technique that would "confidently identify Cm strains to the subspecies level". Not only did each subspecies have a unique fingerprint but strains belonging to the same subspecies produced fingerprints that were "identical or nearly identical" to each other. This method is practical for diagnosing plant pathogens not only because it is a reliable method to distinguish bacteria to the subspecies level, but also because whole cells can be used in the extraction procedure including cells that are extracted from symptomatic plant tissue. Differentiation of the 5 subspecies of Cm was also done using the BOX-A1R primer which is another method using repeated DNA

sequences. This technique is not labor intensive and can be completed in just two working days (Smith et al., 2001).

Using a TaqMan-PCR protocol, differentiation of each subspecies can be made in a single PCR run. A primer master mix can be formulated to isolate and amplify the subspecies of interest dependent on the specific TaqMan probe (Bach et al., 2002)

1.9.3 Other Diagnostic Techniques

Fatty acid methlyesterase (FAMES) profiles are created by measuring carbon evolution using gas-liquid chromatography (Gitaitis et al., 1991).

Carbon substrate utilization using inoculated microarray plates has been successful in detecting a wide array of both gram-negative and gram-positive bacteria (Morgan, et al., 2009; ***). This technique, however, has been shown to be less reliable for the identification of gram-positive, coryneform, phytopathogens (Harris-Baldwin and Gudmestad, 1996; Kaneshiro et al., 2006).

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Table 1. List of *Clavibacter michiganensis* subspecies and the diseases caused on their respective host plant.

Subspecies	**Host Plant**	**Disease**
C. m. insidiosus	Alfalfa (*Medicago sativa*)	wilt of alfalfa
C. m. michiganensis	Tomato (*Lycopersicon esculentum*)	wilt and canker
C. m. nebraskensis	Corn (*Zea mays*)	wilt and leaf blight
C. m. sepedonicus	Potato (*Solanum tuberosum*)	wilt and ring rot of tuber
C. m. tessellarius	Wheat (*Triticum aestivum*)	leaf freckles and spots

Chapter II

Evaluation of the specificity and selectivity of commercially available serological diagnostic tests for the detection of *Clavibacter michiganensis* subsp. *nebraskensis*.

2.1 INTRODUCTION

A reliable, accurate, cost effective and quick diagnostic tool is needed for the detection of Cmn in corn. Relying on semi-selective media to isolate and detect Cmn is difficult because of the myriad of epiphytic and endophytic gram-positive bacteria that are able to grow on CNS (Gross and Vidaver, 1978) and the completion of Koch's postulates can be very time consuming. Furthermore, as seen with the potato pathogen *Clavibacter michiganensis* subsp. *sepedonicus*, the absence of foliar symptoms does not imply the absence of the pathogen (Franc, 1999). Although latency studies have not been conducted with Goss's bacterial wilt, the presence of Cmn has been detected in symptomless corn plants because of the ability of the bacteria to establish epiphytic colonization (Beattie and Marcell, 2002). Surface sterilization of suspect corn tissue may inhibit the ability of any epiphytic organism to remain viable but does not destroy any antigenic properties of that organism; therefore the possibility of cross reactions with epiphytes is not eliminated simply by sterilizing the plant tissue prior to extraction. The objective of this study was to determine the cross-reactiviy and sensitivity of several serological and commercially available tests for the detection of *Clavibacter michiganensis* subspecies, namely Cmn.

2.2 MATERIALS AND METHODS

2.2.1 Preparation of Cultures for Testing

All experiments were conducted in the spring and summer of 2009. Lyophilized isolates of *Clavibacter michiganensis* were re-suspended and transferred to Nutrient Broth Yeast (NBY) agar. Cultures were stored at 4 C. To maintain viable cultures, isolates were transferred to fresh NBY and allowed to grow for 72 h. Single, orange, round colonies were selected and transferred with a sterile cotton swab into micro-bead tubes (Microbank, Pro-Lab Diagnostics, Richmond Hill, ON, Canada) for long term storage at -80 C (Vidaver, 1977).

New cultures were isolated from symptomatic corn leaves that were submitted to the UNL Plant and Pest Diagnostic Clinic from various counties throughout Nebraska in 2008, including one sample that was sent from Indiana. The extraction procedure involved cutting a five cm^2 piece of tissue from the margin of a lesion on a leaf that had symptoms resembling those caused by Goss's leaf blight. The leaf sample was then rinsed in 10% bleach for 90 s followed by a 30 s rinse in sterile distilled water. The tissue was then macerated in a sterile petri dish with approximately 50 ml of sterile distilled water. A loopful of the suspension was streaked onto two separate plates of CNS media (Gross and Vidaver, 1978). Three cultures isolated by the aforementioned extraction procedure (Indiana, 200800559, and 200800600) were later transferred to micro-beads and added to the list of isolates to be tested. There was a total of 32 Cm isolates; 15 Cmn, 5 Cmm, 5 Cmt, 4 Cms and 3 Cmi (Table 1.)

To ensure adequate bacterial titers, each isolate was grown on fresh NBY and single colonies were transferred to individual 250 ml Erlenmeyer flasks containing 125 ml of sterile NBY liquid broth. The flasks were then placed on an agitator for 72 h at 140 rpm at 27 C.

After 72 h of agitation, a dilution series was performed for each isolate in order to count the number of colony forming units (CFU) contained in each flask. 1:10, 1:100, 1:1000,and 1:10000 serial dilutions were made and 100 µl of each dilution was transferred to a plate of NBY agar. A glass stir rod was used to evenly distribute cells across the plate. The cells were allowed to grow at 27 C for 72 h, after which time the visible colonies were counted for each plate and the CFU/ml of the stock solutions was calculated. This process was repeated for each isolate for four replications.

The average concentration for each isolate was approximately 10^5 to 10^6 CFU/ml. A preliminary test conducted with diluted stock solutions revealed that bacterial titers of 10^4 CFU/ml or lower were insufficient to elicit a positive response for any of the ELISAs (data not shown). Therefore, all isolates were tested at concentrations of 10^5 to 10^6 CFU/ml.

2.2.2 Testing Cultures

ELISA. ELISA test kits developed for the detection of Cmm, Cmt and Cms (Agdia, Inc., Elkhart, IN) were used to test each Cm subspecies for cross-reactions. Also, an ELISA developed for the detection of Cmn (Neogen, Inc., Scotland, UK) was used to test each Cm subspecies. ELISA's were conducted according to the company's recommended protocols. For each ELISA, two wells were loaded with 100 µl of stock solution from each isolate. Duplicate wells on each ELISA were filled with 100 µl of a positive control supplied by Agdia and Neogen. Positive controls consisted of lyophilized bacteria provided by each company. For Cmm ELISAs, lyophilized cultures of Cmm were rehydrated and loaded into duplicate wells. The same was done for each ELISA. Negative control well consisted of GEB4 general extract buffer (Agdia, Inc.,

Elkhart, IN) loaded into two wells for the Cmm, Cmt, and Cms ELISAs and general extract buffer 10x (Neogen, Inc., Scotland, UK) loaded into two wells for the Cmn ELISA.

Positive results were determined by calculating the minimum absorbance value as described by Ziems et al., 2007. The minimum absorbance value is calculated by taking the mean of the negative controls times two. The resulting number is then multiplied by 0.1. The average values for duplicate sample wells from isolates that were above this value were considered positive. Plates were read with the OPTImax Tunable Microplate Reader (Molecular Devices Corporation, Sunnyvale, CA) at 405 nm. Test efficiencies were calculated by taking the number of positive tests divided by the total number of tests conducted for a particular isolate (Gudmestad et al., 2009). ELISA tests that resulted in four positive tests out of four tests conducted for a particular isolate were considered to be 100% efficient for that isolate. ELISAs that resulted in three positive tests out of four tests conducted for a particular isolate were considered to be 75% efficient for that isolate, and so on.

ImmunoStrips. Each of the 32 isolates were also tested for a cross reaction using a lateral flow chromatograph (ImmunoStrip®) provided by Agdia for the detection of Cmm. A loopful of bacteria was placed into a microcentrifuge tube containing 0.5 ml of Extraction Buffer SEB4 (Agdia, Inc., Elkhart, IN). The microcentrifuge tube was vortexed for 2 s and an ImmunoStrip was placed into the solution and held for 30 min. Each time the ImmunoStrips were tested a negative control was tested, consisting of the SEB4 extraction buffer only.

In order to determine if cross-reactions are possible to other bacteria outside the species of *C. michiganensis*, 17 isolates were added to the ImmunoStrip study. These isolates represented other common corn pathogens and epiphytes/endophytes that are oftentimes isolated from corn tissue. Other phytopathogenic bacterial were added including some gram-negative plant pathogens (Table 2). Also, 29 additional isolates of Cmn were added to increase sample size and its diversity. These isolates represented the largest temporal and geographical diversity that was available in lyophilized storage. In total, 93 different isolates were tested against the ImmunoStrips. Isolates were plated on NBY agar and allowed to grow and form individual colonies. None of the plates appeared to be contaminated. Colonies were transferred to a glass tube containing 5 ml of extraction buffer using a sterile cotton swab. Bacterial titers were measured using a spectrophotometer. Enough bacteria were added to reach an absorbance of 1.0 ± 0.02 nm read at 640 nm, corresponding to 10^9 CFU/ml. Each solution was then diluted to 10^7 CFU/ml by adding 0.005 ml of solution to a microcentrifuge tube containing 0.495 ml of SEB4 extraction buffer; this resulted in a total volume of 0.5ml. ImmunoStrips were tested by placing them into the resultant 0.5 ml solution at 10^7 CFU/ml. Each time the ImmuoStrips were tested a negative control was tested, consisting of the SEB4 extraction buffer only.

2.2.3 Preparation of Field Samples for ELISA Testing

Forty samples were received at the UNL Plant and Pest Diagnostic Clinic in the summer of 2009 for Goss's bacterial wilt and leaf blight testing. All leaf tissue displayed symptoms typical of the disease (long gray to brown lesions with irregular wavy margins accompanied by discontinuous water-soaked spots and occasionally a glossy sheen

associated with the bacterial exudate). A 1:3 ratio of infected plant tissue to extraction buffer was added to a mesh extraction bag (Agdia, Inc., Elkhart, IN) and macerated. General extract buffer GEB4 (Agdia, Inc., Elkhart, IN) was used to extract plant tissue to be tested with the Cmm, Cmt and Cms ELISAs and general extraction buffer 10x (Neogen, Inc., Scotland, UK) was used to extract plant tissue to be tested with the Cmn ELISA. Extracts were removed from the extraction bags, placed into 1.5 ml microcentrifuge tubes and stored at -20 C.

2.2.4 Testing Field Samples

ELISA. Extracted solutions from each isolate were loaded into each ELISA following the protocol as described in section 2.2.2. For negative controls, GEB4 general extract buffer was loaded into two wells for the Cmm, Cmt, and Cms ELISAs and general extract buffer 10x was loaded into two wells for the Cmn ELISA. Samples were placed back into -20 C storage and tested with the ELISAs one additional time.

ImmunoStrips. ImmunoStrips testing was conducted by placing them into 1.5 ml of the excess extract collected after the above extraction assay was completed. This was done at the time of extraction and repeated when the samples were taken out of -20 C storage to be tested with the ELISAs a second time. Each time the ImmunoStrips were tested a negative control was tested, consisting of the SEB4 extraction buffer only.

2.2.5 Koch's Postulates

Each of the 15 Cmn cultures discussed in section 2.2.1 were transferred to fresh NBY agar and allowed to grow for seven days. For the preparation of inoculum, a sterile cotton swab was used to transfer single colonies of each isolate onto fresh NBY agar. The plates were streaked in a confluent manner across the entire surface of the agar to

ensure adequate growth of each bacterial colony. After five days each plate was filled with sterile distilled deionized water and the bacteria were suspended with a sterile cotton swab. This suspension was added to 200 ml of sterile distilled deionized water. The concentration was determined by testing 3 ml of suspension from each isolate with a spectrophotometer at 640 nm. Five Golden Cross Bantam sweet corn plants were thinned to three plants per 15 cm clay pot in the greenhouse and inoculated at growth stage V3-V4 (Ritchie et al., 1997).

Plants were inoculated by wounding the leaf apex of the fifth leaf from the bottom (including the cotyledon) with a scissors. Two cuts were made on either side of the midrib parallel to the veins (Figure 1). A mark was made with a permanent marker approximately 6.5 cm from the leaf apex. No wounding occurred beyond this point. Immediately after wounding the leaf apex of each of the three plants was suspended in the 200 ml of inoculum to the mark and held for five seconds (Figure 2).

After 10 days, inoculated plants displayed symptoms identical to Goss's leaf blight. Leaf tissue adjacent to the scissor cuts became water-soaked and discontinuous water-soaked spots appeared around the wounded area and progressed toward the proximal end of the leaf, past the mark made 6.5 cm from the apex. Infected leaves were harvested and placed into clear plastic bags. Samples were stored at 4 C. Extractions from leaf tissue revealed bacterial colonies consistent in morphology to Cmn. The extraction procedure used to isolate the bacteria is described in section 2.2.1.

2.2.6 Testing Infected Plant Tissue after Koch's Postulates

ELISA. Bacteria were extracted from the infected tissue according to the Agdia and Neogen protocols as described previously in section 2.2.3. The fifth leaf from the

bottom of a non-inoculated control plant was included as a negative control. A 3:1 ratio of extract buffer and plant tissue was added to a mesh extraction bag and the tissue macerated. The resultant suspension was collected for each strain in 1.5 ml microcentrifuge tubes and stored at -20 C. A suspension of 100 µl was added to two separate wells in the ELISA plate for each of the four replications. For a negative control 100 µl of extract from the non-inoculated control plants was added to two separate wells. Two wells were also filled with 100 µl of extract buffer. Minimum absorbance values were calculated with all four negative control wells.

ImmunoStrips. ImmunoStrips were tested by placing them into the suspension of leaf tissue extract. This was done on two separate occasions and each time a negative control was used. The negative control was the extract collected from the non-inoculated corn leaves discussed in section 2.2.8.

2.3 RESULTS

2.3.1 Test Results for Cultures

ELISA. *C. michiganensis* subsp. *nebraskensis* isolates (with the exception of isolates 20033, 200800559 and 200800600) were positively identified four out of four times when tested with Cmn, Cmm and Cmt ELISAs at a concentration of 10^5-10^6 CFU/ml. ELISAs for the detection of Cms conducted at 10^5-10^6 CFU/ml produced variable results (Table 3).

All Cmm isolates tested positive each time they were tested with the ELISAs developed for the detection of Cmn, Cmm, and Cmt. Cmm isolates tested with the ELISA developed for the detection of Cms were positive only two of the four times or less. All Cmt isolates tested positive each time on the ELISAs developed for the

detection of Cmn and Cmm but results were variable when tested with the ELISAs developed for the detection of Cmt and Cms. Isolates 7621 and 82008 only gave positive results 75% of the time when tested with the ELISA designed for the detection of Cmt. Efficiencies for the detection of Cms and Cmi were poor regardless of the ELISA used (Table 4).

ImmunoStrips. ImmunoStrip tests were positive for each isolate of *C. michiganensis* 100% of the time (Tables 3, 4 and 6). There were only two exceptions, isolates CN72-30 and CN72-33 were both negative 100% of the time (Table 6). These two isolates, however, did not elicit a positive response when tested with Koch's Postulates and were thus not considered to be Cmn. ImmunoStrip tests were negative when tested against bacteria not belonging to the genus *Clavibacter* (Table 7). Negative control samples consisting of only the extraction buffer were negative 100% of the time. There were no false positives when testing the ImmunoStrips against negative controls.

2.3.2 Results from ELISA Testing With Infected Plant Tissue After Koch's Postulates

Eleven of the 15 isolates tested on the Cmn ELISA tested positive with 100% efficiency. Isolates 20033 and 200800559 did not elicit a positive response when tested with the Cmn ELISA. Ten out of the fifteen isolates tested on the Cmm and Cmt ELISAs were positive for a cross-reaction 100% of the time. Isolates 20033, 200800559 and 200800600 gave positive results 50% of the time or less when tested with the Cmm and Cmt ELISAs. The Cms ELISA plate did not cross react with any of the extracts from the infected leaf tissue (Table 3).

2.3.3 Test Results for Field Samples

ELISA. All 40 symptomatic plant samples suspected to have Goss's bacterial wilt and blight tested positive during both replicate tests on the Cmn, Cmm and Cmt ELISAs but tested poorly on the Cms ELISA (data not shown). However, only 15 of the 40 field samples are discussed since Koch's Postulates were performed on only these 15 (Table 5).

ImmunoStrips. All 15 field samples tested positive with the ImmunoStrips. Testing of negative controls always gave negative results (Table 5).

2.4 DISCUSSION

2.4.1 *C. michiganensis* subsp. *nebraskensis*. Cultures

ELISAs developed for the detection of Cmn consistently give positive results when testing Cmn isolates. ELISAs developed for the detection of Cmm and Cmt can also be used to consistently cross react with Cmn isolates. Contamination of the stock solutions of isolates 20033, 200800559 and 200800600, could have resulted in their poor test efficiencies; after plating the stock solutions onto NBY two colonies formed with different and distinct morphologies. These plates contained colonies representative of Cmn and a separate colony that was pale white and flat. This could explain the poor efficiencies of the ELISA tests when these stock solutions were tested. Cmn was still present in the solution, however some other bacterial contaminant was competing for nutrients, thus the concentration of Cmn could have fallen below the detection limit of 10^4 CFU/ml. For isolates 20033 and 200800600, the GEN III Omnilog ID system consistently identified them as *Microbacterium testaceum* and *Pantoea agglomerans* bgp 3, respectively (see Chapter III, section 3.4.1)

The Cms ELISA is designed using a monoclonal capture antibody which is unlike the other ELISA tests used in this study which utilize polycolonal capture and detection antibodies. Monoclonal antibodies tend to be more specific for the antigens for which they are created (De Borer et al., 1988; De Borer and Wieczorek, 1984). The increase in specificity of a monoclonal antibody could explain the inability of the Cms ELISA to consistently detect a cross-reaction from the other subspecies of Cm.

2.4.2 The other *C. michiganensis* subspecies, Cultures.

ELISA plates designed for the detection of *C. michiganensis* subsp. *nebraskensis* seem to react well with Cmn isolates and cross-react well with Cmm and Cmt isolates. These plates, however do not work well for the detection of Cms and Cmi isolates. ELISA plates designed for the detection of *C. michiganensis* subsp. *michiganensis* react well with Cmm isolates and cross-react well with Cmn and Cmt isolates. Again, these plates do not work well for the detection of Cms and Cmi isolates. ELISA plates designed for the detection of *C. michiganensis* subsp. *tesselarius* cross-react well with Cmn and Cmm isolates but not with Cms or Cmi isolates. Cmt isolates 7621 and 80006 were only 75% efficient when tested with the ELISA developed for the detection of Cmt. This could be due to low population numbers that were observed for these two isolates after dilution plating.

2.4.3 ELISA Testing with Infected Plant Tissue after Koch's Postulates

. The results obtained from this part of the study indicate that the Cmn, Cmm and Cmt ELISAs are capable of detecting Cmn by using extracts from infected leaf tissue. Isolates 20033, 200800559 and 200800600 were not detected 100% of the time and these results matched the results from when they were tested using cultures (Table 4). Also,

these three isolates incited symptoms on inoculated plants in the greenhouse that were not as severe as the other 12 isolates tested (data not shown).

2.4.4 ImmunoStrip Testing

Of the 59 strains of Cmn tested with the ImmunoStrips, three strains were negative for each replication. None of the three negative strains caused infection on Golden Cross Bantam when inoculated and were thus considered not to be Cmn. Six other strains did not produce symptoms on Golden Cross Bantam, however they did elicit a positive response when tested with the ImmunoStrips. Isolates may become attenuated after extended periods in storage, so loss of virulence in some isolates is not unexpected (Schuster et al., 1975). Thus we can conclude that the mechanisms for pathogenicity are unrelated to the production of the antibody responsible for the detection of the bacteria.

2.5 LITERATURE CITED

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Table 1. List of the 32 *Clavibacter michiganensis* isolates used to test ELISAs, ImmunoStrips and the GEN III Omnilog ID System.

Bacterial Name	Isolate
C. michiganensis **subsp. *nebraskensis***	**1995** **200716 C** **200714 C** **CN18-1** **CNK-1** **20033** **CNI-1** **CN48-1** **20071-3** **173** **1996** **27822** **200800 559** **200800 600** **Indiana** **(15)**
C. michiganensis **subsp. *michiganensis***	**152-1** **9-21** **CF-2** **140** **JD83-1** **(5)**
C. michiganensis **subsp. *tessellarius***	**7621** **80006** **78181** **82074** **82008** **(5)**
C. michiganensis **subsp. *sepedonicus***	**A5-1** **2535** **331133** **CsMT** **(4)**
C. michiganensis **subsp. *insidiosus***	**P9** **M1B** **P2** **(3)**

Table 2. List of 47 isolates added to test ImmunoStips for cross-reaction.

Bacterial Name	Isolate
Burkholderia andropogonis	**X1131A**
	933 NCPPB
	(2)[a]
Clavibacter michiganensis	**CN18-6**
subsp. *nebraskensis*	**CN38-1**
	CN44-1
	313
	2579 NCPPB
	2581 NCPPB
	CNK-2
	CN81-2
	CN76-1
	CNC-1
	CNSD-1
	CNSD-2
	CN27-11a
	CN72-28
	CN72-30
	CN72-33
	CN72-3A
	CN72-42
	Storm Lake
	1994 NC+
	1996 Pioneer
	13424
	9910
	20037
	31341
	2006 Box Butte
	2006 Sheridan
	2006 Sc Bluff
	9918-1
	(29)
Curtobacterium flaccumfaciens **subsp.**	
flaccumfaciens	**1446 NCPPB**
	6887 ATTC
	(2)
Dickeya zea	**SR61**
	(1)
Endophyte	**017**
	034
	648
	92SaC039
	92Bcc041
	(5)
Erwinia chrysanthemi **var. *zea***	**2001-3**
	(1)
Microbacterium testaceum	**12675**
	(1)
Pantoea stewartii **subsp. *stewartii***	**9A**
	SS104
	(2)

Pseudomonas syringae* pv. *syringae	**2264 NCPPB 475 (2)**
Rathayibacter rathayi	**2574 PDDCC 2576 PDDCC (2)**

[a]The total number of isolates of each species.

Table 3. Efficiency of ELISA and ImmunoStrip tests against *Clavibacter michiganensis* subsp. *nebraskensis* cultures and extracted plant material from each isolate.

	Efficiency of Test (%)									
	Cultures					Extracted From Inoculated Plants				
	ELISA				ImmunoStrip	ELISA				ImmunoStrip
Isolate	Cmn	Cmm	Cmt	Cms	Cmm	Cmn	Cmm	Cmt	Cms	Cmm
1995	100	100	100	25	100	100	100	100	0	100
200716C	100	100	100	50	100	100	100	100	0	100
200714C	100	100	100	50	100	100	100	100	0	100
CN18-1	100	100	100	50	100	100	100	100	0	100
CNK-1	100	100	100	25	100	100	100	100	0	100
20033	100	75	25	25	100	0	0	50	0	100
CN1-1	100	100	100	50	100	100	100	100	0	100
CN48-1	100	100	100	50	100	100	100	100	0	100
20071-3	100	100	100	25	100	100	100	100	0	100
173	100	100	100	50	100	100	100	100	0	100
1996	100	100	100	33	100	100	100	100	0	100
27822	100	100	100	50	100	100	100	100	0	100
200800559	50	75	50	50	100	0	0	0	0	100
200800600	50	50	25	50	100	100	0	0	0	100
Indiana	100	100	100	33	100	100	100	100	0	100
Negative control										0

Table 4. Efficiency of ELISA and ImmunoStrip tests against *Clavibacter michiganensis* subsp. *michiganensis*, *tessellarius*, *insidiosus*, and *sepedonicus* cultures.

		Efficiency of Test (%)				
		Cultures				
		ELISA				ImmunoStrip
Bacterial Name	Isolate	Cmn	Cmm	Cmt	Cms	Cmm
Clavibacter michiganensis* subsp. *michiganensis						
	152-1	100	100	100	50	100
	9-21	100	100	100	25	100
	CF-2	100	100	100	50	100
	140	100	100	100	50	100
	JD83-1	100	100	100	50	100
Clavibacter michiganensis* subsp. *tessellarius						
	7621	100	100	75	25	100
	80006	100	100	100	25	100
	78181	100	100	100	50	100
	82074	100	100	100	0	100
	82008	100	100	75	50	100
Clavibacter michiganensis* subsp. *insidiosus						
	P9	100	100	100	0	100
	M1B	75	75	75	25	100
	P2	0	50	50	25	100
Clavibacter michiganensis* subsp. *sepedonicus						
	A5-1	75	100	75	75	100
	2535	75	75	75	50	100
	33133	75	100	100	75	100
	CsMT	NA	NA	NA	NA	100
	Negative control					0

Table 5. Efficiency of ELISA and ImmunoStrip tests against 15 symptomatic field samples.

	Efficiency of Test (%)				
	Extacted From Field Samples				
	ELISA				ImmunoStrip
Isolate	Cmn	Cmm	Cmt	Cms	Cmm
200900025	100	100	100	100	100
200900406	100	100	100	100	100
200900448	100	100	100	0	100
200900504	100	100	100	50	100
200900530	100	100	100	50	100
200900537	100	100	100	0	100
200900540	100	100	100	100	100
200900541	100	100	100	0	100
200900558	100	100	100	100	100
200900625	100	100	100	100	100
200900626	100	100	100	0	100
200900627	100	100	100	0	100
200900653	100	100	100	0	100
200900686	100	100	100	50	100
200900798	100	100	100	100	100
Negative control					0

Table 6. Efficiency of ImmunoStrip tests against 29 additional *Clavibacter michiganensis* subsp. *nebraskensis* cultures and results from the completion of Koch's Postulates.

Efficiency of Test (%)			
Cultures			
		ImmunoStrip	Koch's
Bacterial Name	Isolate	Cmm	
Clavibacter michiganensis subsp.			
	CN18-6	100	+
	CN38-1	100	+
	CN44-1	100	-
	313	100	-
	2579 NCPPB	100	+
	2581 NCPPB	100	-
	CNK-2	100	-
	CN81-2	100	+
	CN76-1	100	+
	CNC-1	100	+
	CNSD-1	100	+
	CNSD-2	100	+
	CN27-11a	100	+
	CN72-28	100	-
	CN72-30	0	-
	CN72-33	0	-
	CN72-3A	100	+
	CN72-42	100	+
	Storm Lake	100	+
	1994 NC+	100	+
	1996 Pioneer	100	+
	13424	100	+
	9910	100	+
	20037	100	+
	31341	100	+
	2006 Box	100	+
	2006	100	+
	2006 Sc Bluff	100	+
	9918-1	100	+
	Negative control	0	NA
	Positive control	100	NA

Table 7. Efficiency ImmunoStrip tests against 17 bacterial isolates not belonging to the genus *Clavibacter*.

Efficiency of Test (%)		
Cultures		
		ImmunoStrip
Bacterial Name	Isolate	Cmm
Burkholderia andropogonis		
	X1131A	0
	933 NCPPB	0
***Curtobacterium flaccumfaciens* subsp.**		
	1446 NCPPB	0
	6887 ATTC	0
Dickeya zea		
	SR61	0
Endophyte		
	17	0
	34	0
	648	0
	92SaC039	0
	92Bcc041	0
Erwinia chrysanthemi* var. *zea		
	2001-3	0
Microbacterium testaceum		
	12675	0
Pantoea stewartii* subsp. *stewartii		
	9A	0
	SS104	0
Pseudomonas syringae* pv. *syringae		
	2264 NCPPB	0
	475	0
Rathayibacter rathayi		
	2574 PDDCC	0
	2576 PDDCC	0
Negative Control		0
Positive Control		100

Figure 1. Leaf wounding for Koch's Postulates.

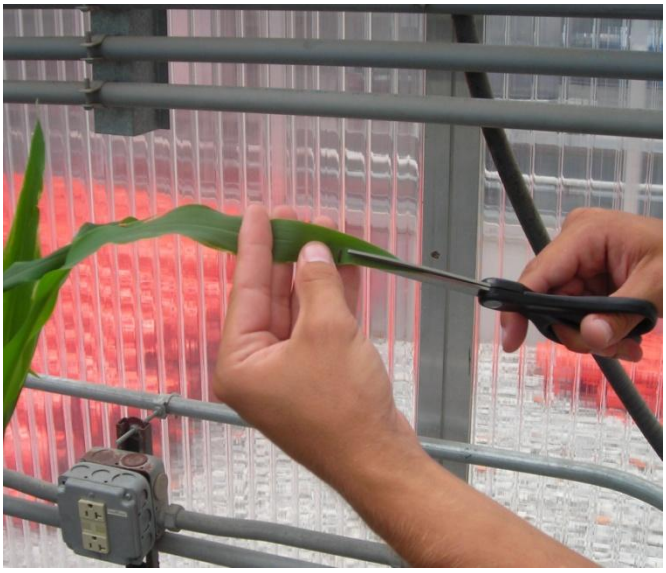
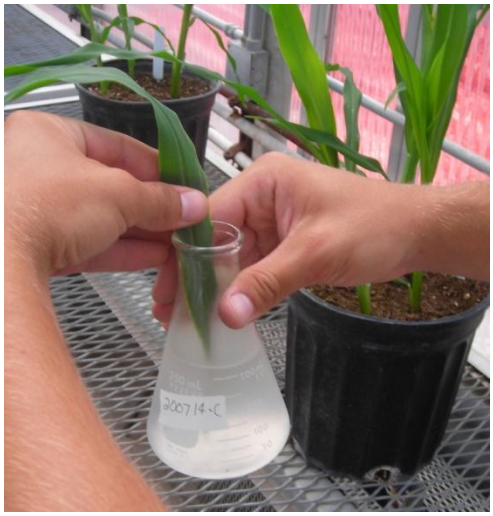


Figure 2. Inoculation of the leaf apex.



Chapter III

Evaluation of the GEN III OmniLog ID system for the detection of *Clavibacter michiganensis* subspecies.

3.1 INRODUCTION

The Biolog GEN III OmniLog Identification system uses carbon substrate utilization profiles to identify over one thousand species of bacteria. A single microarray plate can be used for both gram positive and gram negative bacteria and no pre-tests or follow-on tests are required for identification. The GEN III OmniLog ID system is said to be the gold standard for bacterial diagnostics and has been evaluated for its ability to identify a host of both gram-positive and gram-negative bacteria (Morgan et al., 2006). Many of these studies however have not concentrated on gram-positive coryneform phytopathogens, namely those belonging to the genus *Clavibacter*. Harris-Baldwin and Gudmestad (1996) showed that only 27 to 77% of *Clavibacter* isolates tested were identified to the genus level and even less were identified correctly to the species level. This study was conducted to evaluate the ability of the GEN III OmniLog ID system to identify *Clavibacter michiganensis* subspecies propagated on several different media types.

3.2 MATERIALS AND METHODS

3.2.1 Preparation of Cultures for the GEN III Omnilog ID System

The same 32 *C. michiganensis* isolates (Table 1) used to test ELISAs and ImmunoStrips were used to test the efficiency of the GEN III OmniLog ID System (Biolog, Inc., Hayward CA). All isolates were removed from -80 C storage and propagated on NBY agar at 27 C. All isolates were transferred from NBY to one of the

three media types evaluated in this study and allowed to grow for 72 h. The three media types included: Chocolate agar, Biolog Universal Growth (BUG™; Biolog, Inc., Hayward CA) agar without blood, and trypticase soy agar (TSA).

3.2.2 Inoculation of Microplates for the GEN III OmniLog ID System

Inoculation fluid C and protocol C1 were used when evaluating isolates grown on chocolate agar. It is recommended that protocol C1 be used for slow growing gram-positive cocci and rod shaped bacteria. Cells that had been growing for 72 h were transferred to the inoculation fluid using a tightly bound cotton swab and cell densities were measured. The C1 protocol calls for a 90-98% cell density as measured by transmittance using the Biolog spectrophotometric transmittance machine. For isolates grown on both BUG and TSA agar, inoculation fluid A and protocol A were used to inoculate the microplates. The A protocol requires 90-98% transmittance and is the default protocol used to identify the majority of bacterial species. After inoculation, microplates were incubated for 36 h at 30 C.

3.3 RESULTS

3.3.1 Results From The GEN III OmniLog ID System

Results for the GEN III OmniLog ID system are based on similarity index values (SIM) given to each isolate. Isolates identified with SIM values greater than 0.5 are considered positive and the level of confidence for a particular identification increases as the SIM value approaches 1.00. For SIM values less than 0.5 the GEN III OmniLog ID system lists several bacteria that a particular strain most closely resembles.

C. michiganensis* subsp. *nebraskensis. Isolates propagated on Chocolate agar and evaluated with the C1 protocol resulted in the poorest identifications. Only 45% and

25% of the Cmn isolates were identified to the subspecies level for replications one and two, respectively (Table 2). Furthermore, SIM values were less than 0.5, the minimum value for a proper positive identification. Note that for SIM values less than 0.5, the GEN III OmniLog ID System will not give a positive identification, rather it lists the bacterial species that the carbon utilization profiles most closely resemble.

When TSA agar was used to grow the isolates, SIM values improved but only slightly. One out of the six isolates identified as Cmn in rep one were done so with a SIM value > 0.5 and only two out of the eight isolates identified correctly in rep two had SIM values > 0.5. When propagated on TSA agar the percentage of Cmn isolates identified to the subspecies level was higher; 50 and 67% for replications one and two, respectively (Table 3).

The highest percentages of Cmn isolates identified to the subspecies level were obtained when BUG agar without blood was used to grow isolates prior to inoculating the microplates; 67 and 75% for replications one and two, respectively. When BUG agar without blood was used, 50% of isolates in rep one and 56% of isolates in rep two identified as Cmn were done so with a SIM value > 0.5 (Table 4).

C. michiganensis* subsp. *michiganensis. Only one isolate (CF-2) was ever identified as Cmm. This happened during replication two on chocolate agar. The SIM value was relatively low, 0.312. For the majority of the tests, regardless of the propagation media, Cmm isolates were identified as Cmt and occasionally as Cmn (Tables 5-7).

C. michiganensis* subsp. *tesselarius. Media type played less of a role in the identification of Cmt isolates. The GEN III OmniLog ID System either identified two of

the five or three of the five isolates tested in each replication (Tables 5-7). Also, SIM values for all positively identified isolates were 0.4 or higher with the exception of isolate 80006 tested in replication two with TSA agar as the propagation media (Table 11).

C. michiganensis* subsp. *insidiosus. Only one Cmi isolate was identified as Cmi and the SIM value was low, 0.106. This occurred during replication one with the chocolate agar media source (Table 8).

C. michiganensis* subsp. *sepedonicus. Two Cms isolates were identified as Cms and again SIM values were < 0.5 for each isolate (Table 8).

3.4 DISCUSSION

3.4.1 GEN III OmniLog ID System

The results observed in this study were similar to those obtained in other studies when gram-positive coryneform phytopathogenic bacteria were tested with older versions of the carbon substrate utilization technique from Biolog (Harris-Baldwin and Gudmestad, 1996; Kaneshiro et al., 2006). Harris-Baldwin and Gudmestad, (1996) noticed that the accuracy of identification of isolates improved when the database was supplemented with the profiles of known isolates of a particular species.

A supplemented database may need to be added to the GEN III OmniLog ID System in order to increase the detection of gram-positive coryneform phytopathogenic bacteria. However, this can be both time-consuming and expensive.

Isolates 20033 and 200800600 were consistently and repeatedly identified with SIM values > 0.5 as *Microbacterium testaceum* and *Pantoea agglomerans* bgp 3, respectively, regardless of the media type used for propagation. These results are

consistent with those from the ELISA tests implying that the inoculated microbeads stored at -80 C were contaminated.

Identification of Cmm isolates was poor and matched the results of Harris-Baldwin and Gudmestad, 1996, who found that only three of the 15 *C. michiganensis* subsp. *nebraskensis* isolates tested were positively identified to the subspecies level. Based on the results of these tests, we conclude that the GEN III OmniLog ID system is not a reliable method for accurately identifying *Clavibacter michiganensis* subspecies.

3.5 LITERATURE CITED

1. Harris-Baldwin, A., and Gudmestad, N. C. 1996. Identification of phytopathogenic coryneform bacteria using the Biolog automated microbial identification system. Plant Disease 80:874-878.

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3. Morgan, M. C., Boyette, M., Goforth, C., Volpe Sperry, K., and Greene, S. R. 2009. Comparison of the Biolog OmniLog Identification System and 16S ribosomal RNA gene sequencing for accuracy in identification of atypical bacteria of clinical origin. Journal of Microbiological Methods 79:336-343.

Table 1. List of the 32 *Clavibacter michiganensis* isolates used to test ELISAs, ImmunoStrips and the GEN III OmniLog ID System.

Bacterial Name	Isolate
C. michiganensis **subsp. *nebraskensis***	**1995** **200716 C** **200714 C** **CN18-1** **CNK-1** **20033** **CNI-1** **CN48-1** **20071-3** **173** **1996** **27822** **200800 559** **200800 600** **Indiana** **(15)**
C. michiganensis **subsp. *michiganensis***	**152-1** **9-21** **CF-2** **140** **JD83-1** **(5)**
C. michiganensis **subsp. *tessellarius***	**7621** **80006** **78181** **82074** **82008** **(5)**
C. michiganensis **subsp. *sepedonicus***	**A5-1** **2535** **331133** **CsMT** **(4)**
C. michiganensis **subsp. *insidiosus***	**P9** **M1B** **P2** **(3)**

Table 2. Similarity values and bacterial identifications for Cmn isolates propagated on chocolate agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmn isolates (%) propagated on Chocolate agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *nebraskensis					
	1995	**0.398**	***Clavibacter michiganensis* subsp. *nebraskensis***	**73**	**45**
	200716C	**0.185**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	200714C	**0.226**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CN18-1	**0.096**	***Clavibacter michiganensis* subsp. *sepedonicus***		
	CNK-1	**0.137**	***Gemella bergeri***		
	CNI-1	**0.347**	***Clavibacter michiganensis* subsp. *sepedonicus***		
	[a]CN48-1	**NA**			
	20071-3	**0.168**	***Bacillus pumilus***		
	173	**0.129**	***Clavibacter michiganensis* subsp. *sepedonicus***		
	1996	**0.179**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	27822	**0.312**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	Indiana	**0.219**	***Clavibacter michiganensis* subsp. *sepedonicus***		
Correctly identified Cmn isolates (%) propagated on Chocolate agar. Rep 2.					
Clavibacter michiganensis* subsp. *nebraskensis					
	1995	**0.059**	***Streptococcus orisratti***	**42**	**25**
	200716C	**0.221**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	200714C	**0.094**	***Clavibacter michiganensis* subsp. *sepedonicus***		
	CN18-1	**0.111**	***Bacilius pseudofirmus***		
	CNK-1	**0.103**	***Arcanobacterium phocae***		
	CNI-1	**0.102**	***Gardnerella vaginalis***		
	CN48-1	**0.095**	***Cellulomonas cellasea***		
	20071-3	**0.107**	***Clavibacter michiganensis* subsp. *tessellarius***		
	173	**0.078**	***Gardnerella vaginalis***		
	1996	**0.074**	***Cellulomonas cellasea***		
	27822	**0.433**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	Indiana	**0.115**	***Clavibacter michiganensis* subsp. *nebraskensis***		

[a]Isolate P2 did not grow on Chocolate agar.

Table 3. Similarity values and bacterial identifications for Cmn isolates propagated on TSA agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmn isolates (%) propagated on TSA agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *nebraskensis					
	1995	**0.299**	***Clavibacter michiganensis* subsp. *nebraskensis***	**83**	**50**
	200716C	**0.149**	***Cellulomonas uda***		
	200714C	**0.818**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CN18-1	**0.103**	***Clavibacter michiganensis* subsp. *insidiosus***		
	CNK-1	**0.19**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CNI-1	**0.114**	***Clavibacter michiganensis* subsp. *tessellarius***		
	CN48-1	**0.107**	***Clavibacter michiganensis* subsp. *tessellarius***		
	20071-3	**0.137**	***Cellulomonas uda***		
	173	**0.181**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	1996	**0.785**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	27822	**0.11**	***Clavibacter michiganensis* subsp. *tessellarius***		
	Indiana	**0.074**	***Clavibacter michiganensis* subsp. *nebraskensis***		
Correctly identified Cmn isolates (%) propagated on TSA agar. Rep 2.					
Clavibacter michiganensis* subsp. *nebraskensis					
	1995	**0.202**	***Clavibacter michiganensis* subsp. *nebraskensis***	**83**	**67**
	200716C	**0.168**	***Cellulomonas uda***		
	200714C	**0.817**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CN18-1	**0.299**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CNK-1	**0.098**	***Clavibacter michiganensis* subsp. *tessellarius***		
	CNI-1	**0.373**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CN48-1	**0.29**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	20071-3	**0.133**	***Cellulomonas uda***		
	173	**0.197**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	1996	**0.099**	***Clavibacter michiganensis* subsp. *tessellarius***		
	27822	**0.19**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	Indiana	**0.07**	***Clavibacter michiganensis* subsp. *nebraskensis***		

Table 4. Similarity values and bacterial identifications for Cmn isolates propagated on BUG agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmn isolates (%) propagated on BUG agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *nebraskensis					
	1995	**0.337**	***Clavibacter michiganensis* subsp. *nebraskensis***	**83**	**67**
	200716C	**0.171**	***Cellulomonas uda***		
	200714C	**0.094**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CN18-1	**0.078**	***Rathayibacter tritici***		
	CNK-1	**0.253**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CNI-1	**0.773**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CN48-1	**0.188**	***Clavibacter michiganensis* subsp. *tessellarius***		
	20071-3	**0.661**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	173	**0.242**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	1996	**0.795**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	27822	**0.742**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	Indiana	**0.156**	***Clavibacter michiganensis* subsp. *tessellarius***		
Correctly identified Cmn isolates (%) propagated on BUG agar. Rep 2.					
Clavibacter michiganensis* subsp. *nebraskensis					
	1995	**0.768**	***Clavibacter michiganensis* subsp. *nebraskensis***	**75**	**75**
	200716C	**0.178**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	200714C	**0.81**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CN18-1	**0.211**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CNK-1	**0.34**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CNI-1	**0.812**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CN48-1	**0.108**	***Cellumonas uda***		
	20071-3	**0.163**	***Cellumonas uda***		
	173	**0.193**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	1996	**0.831**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	27822	**0.786**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	Indiana	**0.076**	***Microbacterium lacticum***		

Table 5. Similarity values and bacterial identifications for Cmm and Cmt isolates propagated on chocolate agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmm and Cmt isolates (%) propagated on Chocolate agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *michiganensis					
	152-1	**0.63**	***Clavibacter michiganensis* subsp. *tessellarius***	**100**	**0**
	9-21	**0.528**	***Clavibacter michiganensis* subsp. *tessellarius***		
	CF-2	**0.602**	***Clavibacter michiganensis* subsp. *tessellarius***		
	140	**0.616**	***Clavibacter michiganensis* subsp. *tessellarius***		
	JD83-1	**0.631**	***Clavibacter michiganensis* subsp. *tessellarius***		
Clavibacter michiganensis* subsp. *tessellarius					
	7621	**0.14**	***Staphylococcus sciuri* ss *sciuri***	**80**	**60**
	80006	**0.683**	***Clavibacter michiganensis* subsp. *tessellarius***		
	78181	**0.451**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	82074	**0.697**	***Clavibacter michiganensis* subsp. *tessellarius***		
	82008	**0.633**	***Clavibacter michiganensis* subsp. *tessellarius***		
Correctly identified Cmm and Cmt isolates (%) propagated on Chocolate agar. Rep 2.					
Clavibacter michiganensis* subsp. *michiganensis					
	152-1	**0.429**	***Clavibacter michiganensis* subsp. *tessellarius***	**100**	**20**
	9-21	**0.343**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CF-2	**0.308**	***Clavibacter michiganensis* subsp. *michiganensis***		
	140	**0.424**	***Clavibacter michiganensis* subsp. *tessellarius***		
	JD83-1	**0.292**	***Clavibacter michiganensis* subsp. *tessellarius***		
Clavibacter michiganensis* subsp. *tessellarius					
	7621	**0.422**	***Clavibacter michiganensis* subsp. *nebraskensis***	**100**	**40**
	80006	**0.143**	***Clavibacter michiganensis* subsp. *sepedonicus***		
	78181	**0.176**	***Clavibacter michiganensis* subsp. *tessellarius***		
	82074	**0.697**	***Clavibacter michiganensis* subsp. *tessellarius***		
	82008	**0.128**	***Clavibacter michiganensis* subsp. *insidiosus***		

Table 6. Similarity values and bacterial identifications for Cmm and Cmt isolates propagated on TSA agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmm and Cmt isolates (%) propagated on TSA agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *michiganensis					
	152-1	**0.508**	***Clavibacter michiganensis* subsp. *tessellarius***	**100**	**0**
	9-21	**0.62**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CF-2	**0.26**	***Clavibacter michiganensis* subsp. *tessellarius***		
	140	**0.517**	***Clavibacter michiganensis* subsp. *tessellarius***		
	JD83-1	**0.506**	***Clavibacter michiganensis* subsp. *tessellarius***		
Clavibacter michiganensis* subsp. *tessellarius					
	7621	**0.713**	***Microbacterium testaceum***	**60**	**60**
	80006	**0.504**	***Clavibacter michiganensis* subsp. *tessellarius***		
	78181	**0.116**	***Exiguobacterium acetylicum***		
	82074	**0.567**	***Clavibacter michiganensis* subsp. *tessellarius***		
	82008	**0.501**	***Clavibacter michiganensis* subsp. *tessellarius***		
Correctly identified Cmm and Cmt isolates (%) propagated on TSA agar. Rep 2.					
Clavibacter michiganensis* subsp. *michiganensis					
	152-1	**0.512**	***Clavibacter michiganensis* subsp. *nebraskensis***	**100**	**0**
	9-21	**0.502**	***Clavibacter michiganensis* subsp. *tessellarius***		
	CF-2	**0.372**	***Clavibacter michiganensis* subsp. *tessellarius***		
	140	**0.537**	***Clavibacter michiganensis* subsp. *tessellarius***		
	JD83-1	**0.533**	***Clavibacter michiganensis* subsp. *tessellarius***		
Clavibacter michiganensis* subsp. *tessellarius					
	7621	**0.728**	***Microbacterium testaceum***	**80**	**40**
	80006	**0.302**	***Clavibacter michiganensis* subsp. *tessellarius***		
	78181	**0.203**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	82074	**0.741**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	82008	**0.442**	***Clavibacter michiganensis* subsp. *tessellarius***		

Table 7. Similarity values and bacterial identifications for Cmm and Cmt isolates propagated on BUG agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmm and Cmt isolates (%) propagated on BUG agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *michiganensis					
	152-1	**0.51**	***Clavibacter michiganensis* subsp. *tessellarius***	**100**	**0**
	9-21	**0.562**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CF-2	**0.511**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	140	**0.501**	***Clavibacter michiganensis* subsp. *tessellarius***		
	JD83-1	**0.534**	***Clavibacter michiganensis* subsp. *nebraskensis***		
Clavibacter michiganensis* subsp. *tessellarius					
	7621	**0.742**	***Microbacterium testaceum***	**80**	**60**
	80006	**0.486**	***Clavibacter michiganensis* subsp. *tessellarius***		
	78181	**0.819**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	82074	**0.609**	***Clavibacter michiganensis* subsp. *tessellarius***		
	82008	**0.519**	***Clavibacter michiganensis* subsp. *tessellarius***		
Correctly identified Cmm and Cmt isolates (%) propagated on BUG agar. Rep 2.					
Clavibacter michiganensis* subsp. *michiganensis					
	152-1	**0.508**	***Clavibacter michiganensis* subsp. *tessellarius***	**100**	**0**
	9-21	**0.515**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	CF-2	**0.499**	***Clavibacter michiganensis* subsp. *tessellarius***		
	140	**0.54**	***Clavibacter michiganensis* subsp. *tessellarius***		
	JD83-1	**0.506**	***Clavibacter michiganensis* subsp. *tessellarius***		
Clavibacter michiganensis* subsp. *tessellarius					
	7621	**0.732**	***Microbacterium testaceum***	**80**	**60**
	80006	**0.572**	***Clavibacter michiganensis* subsp. *tessellarius***		
	78181	**0.233**	***Clavibacter michiganensis* subsp. *nebraskensis***		
	82074	**0.61**	***Clavibacter michiganensis* subsp. *tessellarius***		
	82008	**0.507**	***Clavibacter michiganensis* subsp. *tessellarius***		

Table 8. Similarity values and bacterial identifications for Cmi and Cms isolates propagated on chocolate agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmi and Cms isolates (%) propagated on Chocolate agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *insidiosus					
	P9	**0.141**	***Bacillus pseudofirmus***	**33**	**33**
	M1B	**0.106**	***Clavibacter michiganensis* subsp. *insidiosus***		
	P2	**0.148**	***Arcanobacterium phocae***		
Clavibacter michiganensis* subsp. *sepedonicus					
	A5-1	**0.422**	***Staphylococcus pasteuri***	**50**	**50**
	2535	**0.269**	***Clavibacter michiganensis* subsp. *sepedonicus***		
	33133	**0.201**	***Clavibacter michiganensis* subsp. *sepedonicus***		
	CsMT	**0.068**	***Gamella sanguinis***		
Correctly identified Cmi and Cms isolates (%) propagated on Chocolate agar. Rep 2.					
Clavibacter michiganensis* subsp. *insidiosus					
	P9	**0.078**	***Arcanobacterium phocae***	**0**	**0**
	M1B	**0.099**	***Acranobacterium phocae***		
	[a]P2	**NA**	**NA**		
Clavibacter michiganensis* subsp. *sepedonicus					
	A5-1	**0.133**	***Staphylococcus capitis* ss *ureolyticus***	**0**	**0**
	2535	**0.106**	***Bacilllus pseudofirmus***		
	33133	**0.098**	***Acranobacterium bernardiae* (CDC.2)**		
	CsMT	**0.066**	***Arcanobacterium phocae***		

[a]Isolate P2 did not grow on Chocolate agar.

Table 9. Similarity values and bacterial identifications for Cmi and Cms isolates propagated on TSA agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmi and Cms isolates (%) propagated on TSA agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *insidiosus					
	P9	**0.105**	***Gardnerella vaginalis***	**0**	**0**
	M1B	**0.076**	***Gardnerella vaginalis***		
	P2	**0.097**	***Lactobacillus hamsteri***		
Clavibacter michiganensis* subsp. *sepedonicus					
	A5-1	**0.386**	***Staphylococcus warneri***	**0**	**0**
	2535	**0.09**	***Gardnerella vaginalis***		
	33133	**0.098**	***Gardnerella vaginalis***		
	CsMT	**0.096**	***Gardnerella vaginalis***		
Correctly identified Cmi and Cms isolates (%) propagated on TSA agar. Rep 2.					
Clavibacter michiganensis* subsp. *insidiosus					
	P9	**0.102**	***Clavibacter michiganensis* subsp. *tessellariu***	**67**	**0**
	M1B	**0.101**	***Clavibacter michiganensis* subsp. *tessellariu***		
	P2	**0.056**	***Terrimonas ferruginea***		
Clavibacter michiganensis* subsp. *sepedonicus					
	A5-1	**0.505**	***Staphylococcus warneri***	**0**	**0**
	2535	**0.078**	***Lactobacillus hamsteri***		
	33133	**0.095**	***Gardnerella vaginalis***		
	CsMT	**0.065**	***Cellulomonas cellasea***		

Table 10. Similarity values and bacterial identifications for Cmi and Cms isolates propagated on BUG agar. Percent identifications to the species and subspecies level calculated based on the number of correctly identified isolates per number of isolates tested.

Correctly identified Cmi and Cms isolates (%) propagated on BUG agar. Rep 1.					
	Isolate	**SIM**	**ID**	**Species**	**Subspecies**
Clavibacter michiganensis* subsp. *insidiosus					
	P9	**0.126**	***Gardnerella vaginalis***	**33**	**0**
	M1B	**0.088**	***Clavibacter michiganensis* subsp. *tessellarius***		
	***P2**	**NA**	**NA**		
Clavibacter michiganensis* subsp. *sepedonicus					
	A5-1	**0.506**	***Staphylococcus warneri***	**50**	**0**
	2535	**0.089**	***Clavibacter michiganensis* subsp. *tessellarius***		
	33133	**0.08**	***Garderella vaginalis***		
	CsMT	**0.098**	***Clavibacter michiganensis* subsp. *tessellarius***		
Correctly identified Cmi and Cms isolates (%) propagated on BUG agar. Rep 2.					
Clavibacter michiganensis* subsp. *insidiosus					
	P9	**0.144**	***Clavibacter michiganensis* subsp. *tessellarius***	**67**	**0**
	M1B	**0.148**	***Clavibacter michiganensis* subsp. *tessellarius***		
	[a]P2	**NA**	**NA**		
Clavibacter michiganensis* subsp. *sepedonicus					
	A5-1	**0.537**	***Staphylococcus warneri***	**25**	**0**
	2535	**0.054**	***Globicatella sanguinis***		
	33133	**0.073**	***Clavibacter michiganensis* subsp. *tessellarius***		
	CsMT	**0.083**	***Lactobacillus hamsteri***		

[a]Isolate P2 did not grow on BUG agar.

Chapter IV

Thesis Conclusions

It can be concluded from this study that the ImmunoStrips developed by Agdia Inc. for the detection of *C. michiganensis* subsp. *michiganensis* will consistently cross react with Cmn and thus provide accurate diagnoses of Goss's bacterial wilt and leaf blight. Furthermore, ImmunoStrips do not require the use of lab technologies and the procedure may be carried out in the field for immediate diagnosis. Because of their ease of use and relative low cost, ImmunoStrips are a very attractive option for crop consultants and diagnosticians for the identification of Goss's bacterial leaf blight and wilt in corn. One caveat however, is that these test strips will cross react with any of the other four subspecies of Cm. It is possible that one subspecies of Cm might be present on the leaf surface of the host of a different Cm subspecies in cropping systems that involve the association of two different Cm host crops. For example, corn produced in the western part of Nebraska is often grown in close association to potatoes, sometimes even in the same field. Given what we know about the persistence of these bacteria in plant debris contained in the soil, it is not unrealistic to expect to find Cms living epiphytically on the leaves of corn and vice versa.

The Cmn, Cmm and Cmt ELISAs are also a reliable tool for the detection of Cmn in infected leaf tissue. Given their ability to cross react with the other Cm subspecies, the same caveat for the ImmunoStrips can be extended to the ELISAs. It has been clearly shown that the ELISAs do have a detection limit somewhere around 10^4 CFU/ml. Therefore, adequate cell concentrations need to be obtained for these tests otherwise there is a chance for false negatives as the result of low bacterial titers.

The GEN III Omnilog ID System is capable of expanding its database by entering the profiles of known isolates. As discussed in Chapter three, supplemented databases have been shown to improve the accuracy of Cmn identifications. However, it is not recommended that the GEN III Omnilog ID System be used to identify any Cm subspecies given the current database.

Appendix

5.1 Preparation of CNS Media

The recipe used to make CNS selective media was adapted from the "Laboratory Guide for Identification of Plant Pathogenic Bacteria, Third Edition". The only change involved substituting Bravo 6F® (Syngenta Inc., Wilmington, DE) for Daconil 2787-F (Diamond Shamrock Corp., Cleveland, OH). Both of these fungicides utilize the same active ingredient, chlorothalonil. From the stock solution of Bravo 6F which is 54% a.i., a two percent solution was made with sterile distilled deionized water. Instead of using Daconil according to the recipe, 62.5 µl of the two percent Bravo solution is added to the medium.

5.2 Initial Results Regarding Cmm ELISA Spiked Minimum Absorbance Values

Minimum absorbance values for the Cmm ELISA were spiked when negative controls consisting of uninfected corn leaf tissue were tested (Table 1). This resulted in false negatives for each isolate tested except isolates 173, and CN48-1. Similar results were obtained when testing this ELISA for the detection of *C. michiganensis* subsp. *michiganensis* in tomato plants with uninfected tomato plant extract as a negative control (Agdia personal contact). Agdia suggested a boiling protocol in which leaf samples were boiled in sterile distilled water for 10 minutes. If a leaf compound interaction occurs with the ELISA that raises the absorption values of the negative controls, the interaction does not affect Cmn and Cmt ELISAs.

This experiment was repeated and the minimum absorbance values were not spiked. Also, I was unable to observe elevated minimum absorbance values when the

Cmm ELISA was tested with uninfected plant material in several follow up experiments (data not shown). Non-inoculated leaves of Golden Cross Bantam were extracted following Agdia's protocol for ImmunoStrip testing. Extracts were tested on the Cmm ELISA along with positive controls. The idea was that the plant material used in the ELISA would cause spiked minimum absorbance values and thus the positive controls would appear negative. However, in each of the four replications, minimum absorbance values were low (< 0.2 nm) and all of the positive controls were read as positive.

5.3 LITERATURE CITED

1. Schaad, N. W., Jones, J. B., and Chun, W. 2002. Laboratory guide for identification of plant pathogenic bacteria, 3rd edition. APS Press, St. Paul, MN. da Silva, A.C.R. et al. 2002

Table 1. Efficiency of ELISA and ImmunoStrip tests against *Clavibacter michiganensis* subsp. *nebraskensis* cultures and extracted plant material from each isolate.

	Efficiency of Test (%)									
	Cultures					Extracted From Inoculated Plants				
	ELISA				ImmunoStrip	ELISA				ImmunoStrip
Isolate	Cmn	Cmm	Cmt	Cms	Cmm	Cmn	Cmm	Cmt	Cms	Cmm
1995	100	100	100	25	100	100	0	100	0	100
200716C	100	100	100	50	100	100	0	100	0	100
200714C	100	100	100	50	100	100	0	100	0	100
CN18-1	100	100	100	50	100	100	0	100	0	100
CNK-1	100	100	100	25	100	100	0	100	0	100
20033	100	75	25	25	100	0	0	50	0	100
CN1-1	100	100	100	50	100	100	0	100	0	100
CN48-1	100	100	100	50	100	100	100	100	0	100
20071-3	100	100	100	25	100	100	0	100	0	100
173	100	100	100	50	100	100	100	100	0	100
1996	100	100	100	33	100	100	0	100	0	100
27822	100	100	100	50	100	100	0	100	0	100
200800559	50	75	50	50	100	0	0	0	0	100
200800600	50	50	25	50	100	100	0	0	0	100
Indiana	100	100	100	33	100	100	0	100	0	100
Negative control										0