Development of an Immunosorbent Assay for Seedborne *Erwinia stewartii* in Corn Seeds

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ABSTRACT

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Specificity of polyclonal and monoclonal antibodies generated to *Erwinia stewartii* was determined by testing 167 bacterial strains in enzyme-linked immunosorbent assay (ELISA). Of these, the antibodies were positive to all 43 *E. stewartii* strains tested. Reaction of the monoclonal antibody to all other bacterial strains was negative. However, the polyclonal antibodies reacted with seven of 105 nonpathogenic bacteria from corn plants and seeds determined not to be virulent *E. stewartii*. A double-sandwich ELISA that used the polyclonal and monoclonal antibodies was developed to detect *E. stewartii* in ground corn-seed samples. A comparison of four ELISA procedures to detect *E. stewartii* in pure culture and mixed with corn-seed tissue revealed that the most appropriate procedure was a double-sandwich ELISA using polyclonal antibodies for capture and monoclonal antibodies for detection. The assay detected *E. stewartii* antigen in seeds from plants inoculated with a rifampicin and nalidixic acid tolerant strain of *E. stewartii* but not in seeds from uninoculated plants. The presence of viable *E. stewartii* in seeds from inoculated plants was confirmed by culture. Analyses of 400 single seeds showed an absolute positive correlation between recovery of bacteria and ELISA response in eight seeds. *E. stewartii* was recovered from 10 other seeds that had a negative ELISA response. Recovered bacterial populations in nine of these 10 seeds were below the threshold of detection by ELISA.

Additional keywords: serology, Stewart's bacterial wilt.

Erwinia stewartii is the causal agent of Stewart's bacterial wilt (SBW) of corn (*Zea mays* L.). The disease caused severe economic losses in the United States in the 1930s but, since then, has been effectively controlled by resistant hybrids (13). Occasional outbreaks of the disease have occurred in recent years in Canada (1) and Italy (2).

The bacterium overwinters in mature corn flea beetles (*Chaetocnema pulicaria* Melsh.), which feed on young corn plants in the spring and infect them with *E. stewartii*. The disease tends to be more prevalent after unusually warm winters, which improve the survival rate of the corn flea beetle vector (16).

SBW also has been reported to be seed-transmitted (11,13), although direct evidence for this is limited to laboratory studies. Despite the lack of evidence for seed transmission in the field, more than 50 countries have phytosanitary restrictions for seedborne *E. stewartii* on corn seed to avoid introduction of the pathogen. Export restrictions require either a field inspection of the growing crop for symptoms of SBW or a laboratory seed health test. The current laboratory test, a grow-out procedure, is based on the assumption that seedborne *E. stewartii* is transmitted to seedlings on which detectable symptoms will be expressed. Although this has been established on agar slants (6) and in greenhouse grow-out tests (14), transmission has not been demonstrated in the field (11). Serological techniques offer a possible means for developing a rapid and more reliable seed health test for this pathogen and could be used to further study the potential role of seedborne *E. stewartii* in the disease cycle. Consequently, the objective of this study was to develop a rapid and reliable immunosorbent assay for *E. stewartii*.

MATERIALS AND METHODS

Bacterial strains. Bacteria used in this study, isolated from many locations in the United States, included strains of *E. stewartii* from culture collections at Ohio State University and Iowa State University. Also included were 28 wild-type strains of *E. stewartii* (SW87-1–SW87-28) obtained from corn leaves showing Stewart's wilt symptoms in production fields in Iowa in 1987, a series of unidentified bacterial strains from corn leaves and corn seed originating in Iowa, and various other plant-associated bacterial strains from culture collections at Iowa State University (Table 1).

A nalidixic acid and rifampicin resistant strain of *E. stewartii* (9A) was obtained by screening a nalidixic acid resistant strain of *E. stewartii* (DC283 obtained from D. Coplin, Ohio State University) for a mutant that was also rifampicin-resistant. Strain DC283 was grown on nutrient agar (Difco Laboratories, Detroit, MI) containing 0.05% antifoam B (Sigma Chemical Co., St. Louis, MO) at room temperature for 24 h and suspended in phosphate-buffered saline (0.02 M sodium phosphate, 0.85% NaCl, pH 7.2; PBS). Aliquots of 0.1 ml of suspension were spread on gradient plates of nutrient agar overlaid with nutrient agar amended with 50 mg of rifampicin per liter (Sigma) and 1% dextrose. Colonies were transferred to nutrient agar plates either unamended or amended with 25 mg of nalidixic acid per liter (Sigma). Colonies developing on both media were considered rifampicin and nalidixic acid resistant, but not rifampicin or nalidixic acid dependent.

Pathogenicity tests. All bacterial strains were tested on corn seedlings of either the dent corn inbred A632 or the sweet corn hybrid Hybrid Pride of Canada (HPC) grown in the greenhouse in pasteurized soil. Cultures were grown on nutrient agar (Difco Laboratories, Detroit, MI) containing 0.05% antifoam B (Sigma Chemical Co., St. Louis, MO) for 48–72 h and then suspended in sterile PBS. A sample of 0.10 ml of a cloudy bacterial suspension (about 10^9–10^{10} cfu/ml) was injected into the pseudostem approximately 1–2 cm above the soil line at the four- to five-leaf stage (3,4). A virulent strain (SS104 or 9A) of *E. stewartii* was used as a positive control and sterile PBS was used as a negative control. The corn seedlings were inspected periodically for 14 days for characteristic symptoms of SBW.

Biochemical tests. Biochemical profiles of selected bacterial strains were determined by using the API 20E System (API Analy-

tab Products, Division of Sherwood Medical, Plainview, NY). Bacteria were grown for 24 h on nutrient agar. Sterile applicator sticks were used to transfer bacteria by touching single colonies and swirling in 5 ml of sterile saline in a test tube. Suspensions from two colonies from each bacterial strain were thus prepared in separate tubes. The bacterial suspensions were then added to the cupules on separate API 20E System strips. The strips were incubated at 30 C for 18–24 h and then evaluated according to the manufacturer's recommended procedures (12).

TABLE 1. Bacteria screened by enzyme-linked immunosorbent assay

Bacterium	Designation/Source	Collection[a]
Erwinia stewartii	SS104, Illinois, 1967, ICPB[b]	OSU
Erwinia stewartii	AS2, Iowa	ISU
Erwinia stewartii	1303, Iowa	ISU
Erwinia stewartii	DC283, spontaneous SS104 Nal^r mutant, ICPB	OSU
Erwinia stewartii	DC160, New York, 1976, T. Woods ES-3	OSU
Erwinia stewartii	SW1, Ohio, 1974	OSU
Erwinia stewartii	SW2, Ohio, 1974	OSU
Erwinia stewartii	SW13, Indiana, 1974	OSU
Erwinia stewartii	SW14, Indiana, 1974	OSU
Erwinia stewartii	SW19, Kentuky, 1975	OSU
Erwinia stewartii	SW36, Tennessee, 1975	OSU
Erwinia stewartii	SW39, North Carolina, 1975	OSU
Erwinia stewartii	SW45, Illinois, 1975	OSU
Erwinia stewartii	GAL8, EPS spontaneous avirulent mutant	OSU
Erwinia stewartii	MU14110, acapsular avirulent mutant (Mu pf7701)	OSU
Erwinia stewartii	Mu51, capsular avirulent mutant (Mu pf7701)	OSU
Erwinia stewartii	SW87-1 to SW87-28, 28 wild type isolates from diseased corn plants, Iowa, 1987	ISU
Miscellaneous identified bacteria		
E. herbicola ssp. *ananas*	ATCC 8366	ISU
E. carotovora ssp. *atroseptica*	ECA-1	ISU
E. carotovora ssp. *carotovora*	ECC495	ISU
E. chrysanthemi	SR58	ISU
Erwinia saprophyte	DC130	OSU
Enterobacter agglomerans	G1B	ISU
E. agglomerans	LM2A	ISU
E. agglomerans	N1646	ISU
E. agglomerans	3135F	ISU
Agrobacterium tumefaciens		ISU
A. radiobacter		ISU
Clavibacter michiganense ssp. *nebraskense*		ISU
Pseudomonas fluorescens		ISU
P. marginalis		ISU
P. phaseolicola		ISU
P. solanacearum		ISU
Xanthomonas campestris pv. *campestris*		ISU
Unidentified bacteria		
16 isolates from corn tissue	Field or greenhouse grown corn plants	ISU
90 isolates from corn seed	Field or greenhouse grown corn plants	ISU
G2168	Unknown origin	ISU

[a] ISU, cultures from collection of E. Braun, Department of Plant Pathology, with the exception of *Enterobacter agglomerans*, which was obtained from the collection of P. Hartman, Department of Microbiology, Iowa State University; OSU, cultures obtained from D. Coplin, Ohio State University.

[b] International Collection of Phytopathogenic Bacteria, University of California, Davis.

Preparation of antibodies. Polyclonal antibodies to *E. stewartii*, strain SS104, were produced in New Zealand white rabbits by immunization with bacterial cells. Live bacterial cells were prepared by growing *E. stewartii* in liquid NMC medium (R. Carlson and A. K. Vidaver, University of Nebraska, *personal communication*) (60 g of Na_2PO_4, 30 g of KH_2PO_4, 10 g of NH_4Cl, 1 g of L-glutamine, 1 mg of nicotinic acid, 0.1 mg of thiamine, 0.01 mg of biotin, 0.2 g of DL-methionine, 0.25 g of $MgSO_4$, and 10 g of glucose per liter of distilled water) and harvesting the cells during logarithmic growth phase by centrifugation at 12,100 *g* for 10 min. Bacteria were resuspended in sterile PBS, washed three times by centrifugation, and resuspended in PBS.

Primary immunization was by a subcutaneous injection of 10^9 bacteria (determined by optical density at 540 nm) in 1 ml of PBS emulsified in 1 ml of Freund's complete adjuvant. Subsequent subcutaneous injections of the bacteria were made at 3, 6, 10, 16, 29, and 41 wk but were prepared in Freund's incomplete adjuvant. Hyperimmune serum was collected from the rabbits approximately 10 and 20 days after each injection. Antibodies were purified by a series of three ammonium sulfate precipitations and protein A affinity chromatography (7,17). Preimmune serum, collected before the first immunization, was used as a negative control.

For production of monoclonal antibodies, live cells of *E. stewartii* were prepared as described. A 0.1-ml suspension of the bacteria (1×10^7 cfu/ml) was injected intraperitoneally into separate BALB/c mice. Three weeks later, mice that tested positive for antibody production by an indirect ELISA were injected in the tail vein with 10^6 cells of *E. stewartii* in 0.1 ml of sterile PBS. After 3 days, mouse spleen cells were fused with Sp2/O-Ag14 myeloma cells, as described previously (18). The primary hybridomas, and all cloned cell lines, were screened for antibody production by indirect ELISA. Antibody-secreting hybridomas were cloned by limiting dilution. Cloned hybridomas that produced antibodies that recognized *E. stewartii* cells were increased in culture to produce antibodies for further preliminary screening and isotyping as previously described (5,7,17). Potentially valuable hybridoma cells were harvested from growth media by centrifugation and injected into pristane-primed mice for ascitic fluid production. The monoclonal antibodies were purified by using protein A chromatography (5,7,17).

ELISA procedures. All strains shown in Table 1 were screened in a double-antibody sandwich ELISA procedure using polyclonal antibodies and in an indirect ELISA using monoclonal antibodies. For the indirect ELISA, strains were grown for 48 h on nutrient agar (Difco) containing 0.05% antifoam B (Sigma), and cells were flushed from the surface of the agar with PBS and washed by centrifugation three times. The final bacterial pellet was resuspended in carbonate buffer (0.05 M sodium carbonate, pH 9.6). Cells were bound to the microtiter plate wells by incubating for 1 h at 20 C or overnight at 4 C. The wells were blocked with BLOTTO (5% nonfat dry milk prepared in PBS containing 0.05% Tween 20 [PBS-Tween], 0.03% antifoam A, and 0.02% NaN_3 [9]) and hybridoma culture medium was added. The latter had been diluted with PBS-Tween to an optimum concentration (approximately 1:50) as determined by calculation of the maximum positive/negative (P/N) ratio (7). Alkaline phosphatase-labeled rabbit antimouse IgG (Sigma) was added, followed by the substrate (1 mg/ml of *p*-nitrophenyl phosphate in 10% diethanolamine, pH 9.8).

The polyclonal antibodies used in the double-antibody sandwich ELISA protocol were bound to the microtiter plates in carbonate buffer. Wells were blocked with BLOTTO, and the bacterial samples were resuspended in PBS, pipetted into three wells per plate and replicated over three plates. Alkaline phosphatase-conjugated (7,17) polyclonal antibody was added, followed by the substrate.

Four different ELISA protocols (A, B, C, and D) were compared. Polyclonal antibodies for both the capture antibody and the alkaline phosphatase-conjugated (7,17) second antibody were used in ELISA-A. Monoclonal antibody C/G7/B2 for both the

capture antibody and the alkaline phosphatase-conjugated second antibody was used in ELISA-B. Polyclonal antibodies were used for the capture antibody, unlabeled monoclonal antibody was used for the second antibody, and an alkaline phosphatase-labeled antimouse immunoglobulin (Sigma) was used as a signal antibody in ELISA-C. For these assays, the capture antibody was bound to the wells in carbonate buffer. The wells were blocked with BLOTTO and the test sample added. The conjugated polyclonal and monoclonal antibodies were then added in ELISA-A and ELISA-B, respectively. In ELISA-C, the unlabeled monoclonal antibody was added after addition of the test sample followed by the labeled antimouse immunoglobulin. Finally, substrate was added to the wells in all assays. In the fourth protocol, ELISA-D, the test sample was bound directly to the microtiter plate in carbonate buffer. The wells were blocked with BLOTTO. Unlabeled monoclonal antibody was added, followed by the alkaline phosphatase-labeled antimouse immunoglobulin and the substrate.

All assays were carried out at 20 C in Immulon I plates (Dynatech Laboratories, Inc., Chantilly, VA), which were incubated in each step for at least 1 h. Reaction products, after incubation of wells containing substrate for approximately 1–1.5 h, were determined spectrophotometrically at 405 nm. Volumes of 50 μl per well were used for all reagents except the blocking agent, BLOTTO, which was added at 100 μl per well. Plates were washed three times with PBS-Tween between steps. Optimum concentrations of the immunoreagents were determined by calculation of the maximum P/N (7). Strain SS104 of *E. stewartii* was used as the positive control, and *Agrobacterium tumefaciens* and PBS (polyclonal antibodies) or sodium carbonate buffer (monoclonal antibodies) were used as negative controls. An additional control for the indirect ELISA consisted of a set of wells in which strain SS104 had been absorbed to the wells, but media in which SP2/O cells had been grown, which contained no specific antibodies, were substituted for culture media containing the monoclonal antibodies. The sample mean absorbance was determined by calculating the average of the three plate means (three wells per plate). Sample means greater than twice the mean absorbance of *A. tumefaciens* were regarded as positive.

Production of seeds infected with *E. stewartii*. Sweet-corn plants (HPC) grown in the greenhouse either were inoculated with strain 9A or were not inoculated. Mature ears were harvested, dried in a small laboratory corn drier for 72 h using unheated air (13% moisture or less), and stored at 10 C and 50% relative humidity until needed. Infected seeds were produced in the field from the inbred line A632, which was inoculated in the field at Ames, IA, in 1986 and 1987 with strains 9A or SS104. Ears were harvested, dried, and stored.

Preparation of seed samples for ELISA. Procedures for ELISA detection in bulk corn-seed samples were compared by using seeds harvested from plants of the inbred A632 grown in the field in 1986. Samples of 500 seeds selected at random from plants either inoculated with strain SS104 or from uninoculated plants were surface sterilized for 1 min in 0.5% NaOCl, rinsed three times in sterile distilled water, and soaked overnight at 4 C in 300 ml of PBS containing 0.02% NaN_3. Seed was then ground in a food blender (model 854.38J, Oster Corporation, Milwaukee, WI) for 1 min, the suspension filtered through a single layer of cheesecloth, and a 50-ml subsample of the filtrate centrifuged at 1,085 *g* for 10 min at 5 C. The supernatant was decanted, and the pellet was resuspended in PBS in approximately one-eighth of the previous volume. The ELISA-A protocol was then used to test four 100-μl samples from the PBS soak solution, the ground seed suspension, the supernatant, and the resuspended pellet to determine which sample preparation would yield the maximum P/N.

Detection limits of ELISA protocols. The limits for detection of *E. stewartii* in seeds by ELISA protocols A, B, C, and D were determined by using suspensions of seed tissue amended with a range of concentrations of *E. stewartii*. The seed suspensions were prepared from 40-g samples of corn seeds harvested from plants from the field (A632) and from the greenhouse (HPC) that were not inoculated with bacteria. Seeds were surface sterilized in 70% ethanol for 1 min, rinsed three times in sterile distilled water, and ground for 1 min in 125 ml of PBS containing 0.02% NaN_3 with a Polytron homogenizer (probe PT 10/35; Brinkman Instrument Co., Westburg, NY). The seed suspensions were strained through a single layer of cheesecloth and the filtrates centrifuged as described. The supernatant from each group of seeds was dispensed in 1-ml volumes into two separate sets of test tubes. A third set of tubes contained 1 ml of PBS per tube.

A 10-fold dilution series of strain SS104 was prepared in sterile PBS from a 24-h-old culture. The concentration at each dilution was determined by plating. One milliliter from each bacterial dilution was added to separate tubes containing the two seed supernatants and to a tube containing PBS. Controls included tubes containing the two seed supernatants and PBS, which did not contain strain SS104. Each tube was mixed well and 100-μl samples were transferred from each tube to three wells of a microtiter plate. Assays were performed by using ELISA-A, ELISA-B, and ELISA-C protocols and were replicated in three microtiter plates. The absorbance value above which the bacteria were considered to be detected was established as twice the absorbance of the respective unamended diluents.

In a second experiment, the detection limit of the ELISA-C protocol was compared with that of ELISA-D. Seed tissue was prepared as described, except that, for ELISA-D, the seed supernatant was prepared in sodium carbonate buffer. Additionally, a set of control tubes was prepared for ELISA-D containing 1 ml of sodium carbonate buffer. Strain SS104 was grown and suspended in PBS as described. The suspension was then divided into two equal volumes, and the bacteria were concentrated by centrifugation at 12,100 *g* for 10 min at 5 C. The supernatants were discarded, one pellet was resuspended and serially diluted in sterile PBS for use in ELISA-C, and the other was resuspended

TABLE 2. Assessment of pathogenicity and enzyme-linked immunosorbent assay (ELISA) of selected bacteria by using polyclonal and monoclonal antibodies raised to *Erwinia stewartii*

Source of bacteria	No. of isolates	Pathogenicity[a]	Type of antibody[b]	
			Polyclonal	Monoclonal
E. stewartii				
Culture collection	12	+	+	+
Culture collection	3[c]	−	+	+
From diseased plants	28	+	+	+
Miscellaneous identified bacteria[d]				
Culture collection	17	ND[e]	−	−
Unidentified bacteria				
From corn plants	15	−	−	−
From corn plants	1	−	+	−
From corn seed	83	−	−	−
From corn seed	5	−	+	−
From corn seed (P-53)	1	−	+	+/−
From corn seed (IC-E)	1	+/−	+	+
Unknown origin (G2168)	1	−	+	+

[a] Determined by observation of inoculated corn seedlings in the greenhouse for water soaking and wilt symptoms characteristic of Stewart's bacterial wilt. Plus sign indicates symptoms were observed, and minus sign indicates symptoms were not observed in inoculated corn seedlings.

[b] The polyclonal antibodies were used in a double-antibody sandwich ELISA, and the monclonal antibodies in an indirect ELISA. Bacterial samples were 50 μl per well tested in four wells per plate and replicated on three plates. Plus sign indicates a positive response (greater than 2× the negative control which generally ranged from 0.05 to 0.15, minus sign indicates a negative response, and (+/−) indicates variable results (five of 12 wells gave a marginally positive response).

[c] This group includes Ga18, Mu14110, and Mu51, which have been identified as being avirulent by Dr. David Coplin, Ohio State University.

[d] Identified as not being *E. stewartii*.

[e] Not determined.

and serially diluted in an equal volume of sodium carbonate for use in ELISA-D. The bacterial dilutions were then added to the seed supernatants and control tubes as described. Bacterial populations were determined by plating the bacteria resuspended and serially diluted in PBS.

ELISA tests on bulk seed samples. Seed supernatants were prepared as described from samples of 40 g of seed harvested from uninoculated HPC plants or from HPC plants inoculated with strain 9A. The supernatants were tested by ELISA-C. The supernatant from each sample was pipetted into four wells in a microtiter plate and replicated on three plates. To test for the presence of viable *E. stewartii*, 0.1 ml of each supernatant was spread onto sets of two nutrient agar plates amended (per liter) with 50 mg of cycloheximide; cycloheximide and 25 mg of nalidixic acid; or cycloheximide, nalidixic acid, and 50 mg of rifampicin. Four bacterial strains recovered from seed harvested from inoculated plants and two strains from seed from uninoculated plants were randomly selected and identified. The diagnostic tests used were Gram stain, motility (hanging drop technique), and production of extracellular polysaccharide (colony growth on nutrient agar amended with 1% glucose) (15). Pathogenicity tests, using cultures grown for 72 h and suspended in sterile PBS, as well as the indirect ELISA for screening bacterial isolates using the monoclonal antibody, have been described.

ELISA tests on individual corn seeds. ELISA-C was used to assay single seeds selected at random from samples of 200 seeds harvested from A632 plants inoculated in the field with strain 9A and from HPC plants inoculated in the greenhouse with strain 9A. Seeds were individually surface sterilized in 70% ethanol for 1 min and rinsed three times with sterile distilled water. Each seed was then placed in a sterile 1.5-ml microcentrifuge tube and fractured by squeezing the tube with a pliers. One milliliter of sterile PBS was added to each tube, which was gently shaken for 22 h at 20 C. Samples of 100 μl of the liquid suspension from each tube were pipetted into three wells on a microtiter plate. To test for the presence of viable bacteria, 100-μl samples were also spread on two nutrient agar plates amended with cycloheximide, nalidixic acid, and rifampicin. All bacterial strains recovered were tested for pathogenicity by seedling inoculation and in an indirect ELISA by using the monoclonal antibodies. Controls on each microtiter plate included two sets of wells containing suspensions from two seeds harvested from uninoculated plants. The suspension from the control seeds was spread on one agar plate amended with cycloheximide, nalidixic acid, and rifampicin; on one plate with cycloheximide and nalidixic acid; and on two plates with cycloheximide.

TABLE 3. Biochemical[a] profiles and motility of selected bacterial strains

Test	Bacterial strains[b]						
	SS104	DC283	9A	IC-E	G2168	EHA	AT
ONPG	p[c]	p	p	p	p	p	n
Arginine	n	n	n	n	n	n	n
Lysine	n	n	n	n	n	n	n
Ornithine	n	n	n	n	n	n	n
Sodium citrate	n	n	n	n	n	p	n
Sodium thiosulfate	n	n	n	n	n	n	n
Urea	n	n	n	n	n	n	n
Tryptophan deaminase	n	n	n	n	n	n	n
Tryptophan	n	n	n	n	n	p	n
Sodium pyruvate	n	n	n	n	n	p	n
Charcoal gelatin	n	n	n	n	n	n	n
Glucose	p	p	p	p	p	p	n
Mannitol	p	p	p	p	p	p	n
Inositol	n	n	n	n	n	n	n
Sorbitol	p−	p−	p−	p−	p−	p	n
Rhamnose	n	n	n	n	n	p	n
Sucrose	p	p	p	p	p	p	n
Melibiose	p	p	p−	n	n	p	n
Amygdalin	n	n	n	n	n	p	n
(L+) arabinose	p	p	p	p−	n	p	p−
Oxidase	n	n	n	n	n	n	p
Nitrates	n	n	n	n	n	n	n
Nitrogeneous gas	n	n	n	n	n	n	p
Catalase	p	p	p	p	p	p	p−
Motility	n	n	n	n	n	p	p

[a] API 20E system for the identification of Enterobacteriaceae and other gram-negative bacteria, API Analytab Products, Division of Sherwood Medical, Plainview, NY, used to test the bacterial strains.

[b] *Erwinia stewartii* isolates SS104, DC283, and 9A, a spontaneous Rif$^+$ mutant isolated from DC 283; IC-E, corn seed isolate; G2168, unknown origin; *E. herbicola* ssp. *ananas* (EHA); and *A. tumefaciens* (AT).

[c] p and n designate positive and negative results in their respective tests. p− designates a weak positive test result.

RESULTS

Specificity of antibodies. Preliminary screening of nine monoclonal antibodies by indirect ELISA indicated that the antibodies from hybridoma C/G7/B2 recognized 16 different *E. stewartii* strains and did not cross-react with seven other bacterial genera (data not shown). Antibodies (IgG3, kappa light chain) produced by C/G7/B2 were used in this experiment. In subsequent tests, in which this monoclonal antibody was used in an indirect ELISA, a weak positive reaction (0.015–0.193 above the positive threshold) resulted when high concentrations of several different bacterial isolates were tested. This nonspecific reaction was eliminated by using a standardized bacterial concentration of 10^8 cfu/ml for all test samples.

All strains of *E. stewartii* from the Ohio and Iowa culture collections (Table 1), and those from corn plants with Stewart's wilt symptoms, gave positive responses in ELISA tests with both polyclonal and monoclonal antibodies (Table 2). With the exception of three strains from the Ohio culture collection, all of these strains were pathogenic to corn seedlings. None of the 17 identified strains representing six other bacterial genera originating from plants were positive in either ELISA. All 16 unidentified bacterial strains from corn plants were nonpathogenic and had a negative response in the monoclonal antibody-based indirect ELISA. One of these, however, was positive in the ELISA that used polyclonal antibodies. Eighty-three of the 90 strains from corn seeds were not pathogenic and had negative ELISA responses in both assays. One of the remaining seven strains (IC-E) from corn seed was

TABLE 4. Absorbance values obtained by a double-antibody sandwich enzyme-linked immunosorbent assay (ELISA)[a] of corn seed preparations from plants either inoculated[b] (I) with *Erwinia stewartii* or uninoculated[c] (U)

Seed or control preparation	Absorbance[d]	SD
Soak solution		
I	0.13	0.01
U	0.10	0.01
Ground seed suspension		
I	0.94	0.05
U	0.12	0.02
Supernatant from seed suspension		
I	1.08	0.03
U	0.11	0.01
Resuspended pellet		
I	0.37	0.03
U	0.10	0.01
Controls		
E. stewartii	1.30	0.08
Phosphate-buffered saline	0.12	0.03

[a] The double-antibody sandwich ELISA (ELISA-A) with polyclonal antibodies for capture (15 μg/ml) and labeling with alkaline phosphatase (20 μg/ml) was used.

[b] Five hundred corn seeds harvested from A632 plants inoculated in the field in 1986 with *E. stewartii*, strain SS104.

[c] Five hundred corn seeds from uninoculated A632 plants.

[d] Mean absorbance of five wells at 410 nm. Data is representative of three experiments conducted.

weakly pathogenic on corn seedlings, causing delayed symptoms (3–4 wk) of SBW, and was positive in both assays. The other six strains were nonpathogenic and had a positive ELISA response when polyclonal antibodies were used. Five of these, however, were negative in the monoclonal antibody-based indirect ELISA and one (P-53) alternated between replications from a negative response of 0.11 absorbance units below the positive-negative threshold to 0.20 units above the threshold. Both assays were positive for the nonpathogenic strain G2168, which was of unknown origin.

Biochemical profiles. The biochemical profiles for three *E. stewartii* strains (SS104, DC283, and 9A), which were included in the previous tests, were identical (Table 3). Strain IC-E differed from the *E. stewartii* strains only in its use of melibiose, G2168 differed in use of melibiose and arabinose, and both IC-E and G2168 were nonmotile. *E. herbicola* and *A. tumefaciens* had very different biochemical profiles from *E. stewartii* and were motile. *A. tumefaciens* colonies were white when grown on nutrient agar, whereas the other six strains were yellow to cream-yellow in color.

Preparation of seed samples for ELISA. Absorbance values obtained by the ELISA-A protocol indicated the presence of *E. stewartii* antigen in assays of seed extracts from plants inoculated with strain SS104 (Table 4). The highest ELISA responses occurred in assays of the ground seed suspension and of the supernatant from that suspension. Lower absorbance values resulted from tests of the resuspended pellet, and no signal was evident from assays of the soak solution.

Comparison of ELISA protocols. When twice the absorbance of the corresponding unamended diluent as the lower limit for detection of *E. stewartii* was used, the limits were computed from Figure 1, for *E. stewartii* in PBS alone, PBS plus A632 supernatant, and PBS plus HPC supernatant as (per milliliter) 5.2×10^5 cfu, 6.3×10^5 cfu, and 6.0×10^5 cfu, respectively, for ELISA-A; 4.6×10^6 cfu, 2.5×10^6 cfu, and 3.1×10^6 cfu, respectively, for ELISA-B; and 7.0×10^5 cfu, 1.0×10^6 cfu, and 1.8×10^6 cfu, respectively, for ELISA-C. Detection limits for each of the assays were similar in the presence or absence of seed supernatant in the diluent. ELISA-D, however, detected 5.6×10^6 cfu/ml in sodium carbonate but could not detect bacteria in sodium carbonate containing A632 supernatant or sodium carbonate containing HPC supernatant (Fig. 2). The detection limits for ELISA-C were substantially lower than those for ELISA-B and were slightly greater than those for ELISA-A (Fig. 1).

Detection and recovery of *E. stewartii* from seed samples. The ELISA-C detected *E. stewartii* antigen in bulk samples of seeds harvested from HPC plants inoculated with the *E. stewartii* strain 9A (Table 5). The antigen was also detected in the positive controls, *E. stewartii* strains 9A and SS104, but not in the seeds from plants not inoculated with the bacteria or from the negative controls, *A. tumefaciens*, and PBS. Diagnostic tests on four bacterial strains recovered from the seed harvested from inoculated plants, on agar plates containing cycloheximide, rifampicin, and nalidixic acid (representative of the only colony types which developed), clearly indicated they were *E. stewartii* 9A (Table 6). Numerous bacteria also developed on plates not amended with rifampicin from seeds of plants not inoculated with *E. stewartii*. Few of these showed any similarity to *E. stewartii* colony growth. Two that did were examined further and shown not to be *E. stewartii* based on their lack of extracellular polysaccharide production, nonpathogenicity on corn seedlings, and absorbance values in ELISA similar to those of *A. tumefaciens*, a negative control bacterium.

When the absorbance value of 0.40 was used as the limit above which antigens of *E. stewartii* were considered detected in individual seeds, the ELISA-C protocol detected five and three seeds harvested, from A632 and HPC plants inoculated with strain 9A, respectively (Table 7). Confluent colony growth of *E. stewartii* occurred on rifampicin and nalidixic acid amended plates when each of the ELISA-C positive seed extracts were plated. *E. stewartii* also was recovered from another 10 seeds from the two seed lots with absorbance values lower than 0.40. Nine of these seeds produced fewer than a few hundred *E. stewartii* colonies on the culture plates. Only one gave rise to confluent growth. All 18 strains were confirmed as *E. stewartii* in seedling pathogenicity tests and by indirect ELISA (data not shown). The remaining 382 seeds from inoculated plants and 32 from uninoculated plants had absorbance values lower than 0.40, and no *E. stewartii* colonies were recovered on culture plates.

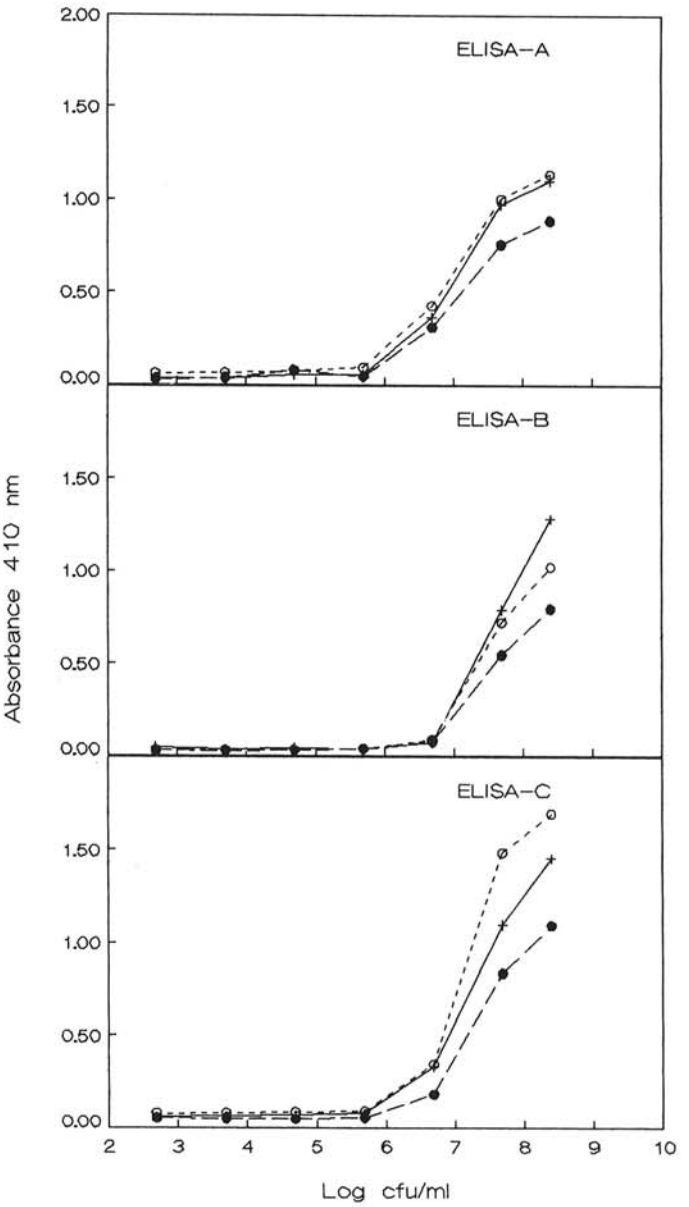

Fig. 1. Absorbance profiles of three double-sandwich enzyme-linked immunosorbent assays (ELISAs) for detection of *Erwinia stewartii* in phosphate-buffered saline (PBS) alone and in PBS amended with corn seed supernatants from inbred A632 and from Hybrid Pride of Canada (HPC). Polyclonal capture antibody (10 μg/ml) and alkaline phosphatase-conjugated polyclonal second antibody (20 μg/ml) were used in ELISA-A. When no bacteria were added to the PBS, PBS plus A632 supernatant, and PBS plus HPC supernatant, the absorbance means were 0.035, 0.074, and 0.035, respectively. Monoclonal antibody C/G7/B2 was used as the capture antibody (30 μg/ml) and alkaline phosphatase-conjugated second antibody (20 μg/ml) in ELISA-B. When no bacteria were added to the PBS, PBS plus A632 supernatant, and PBS plus HPC supernatant, the absorbance means were 0.038, 0.031, and 0.032, respectively. Polyclonal capture antibody (10 μg/ml), monoclonal second antibody (10 μg/ml), and alkaline phosphatase-conjugated antimouse immunoglobulin (1:1,000) was used in ELISA-C. When no bacteria were added to the PBS, PBS plus A632 supernatant, and PBS plus HPC supernatant, the absorbance means were 0.068, 0.086, and 0.059, respectively. Data represent PBS diluent (+ —— +), PBS diluent containing A632 seed supernatant (○- - -○), and PBS diluent containing HPC seed supernatant (● — — ●).

DISCUSSION

Monoclonal and polyclonal antibodies have been produced that, in an ELISA, could detect pure cultures of *E. stewartii* isolated from corn plants and seeds. The polyclonal antibodies showed cross-reactivity with some bacteria isolated from corn plants and seeds. This would seem to limit their use as detection antibodies but would not preclude their use as capture antibodies. The monoclonal antibody cross-reacted with only three bacterial strains from corn plants and seeds. For two of these, IC-E and G2168, biochemical profiles revealed only minor differences from the *E. stewartii* strains tested. Small differences in biochemical profiles of different isolates of the same bacterial genera are common and have previously been reported for *E. stewartii* when the API system was used (12). This similarity to *E. stewartii* in biochemical profiles and in nonmotility, pigmentation, and pathogenicity suggests that IC-E and G2168 are a weakly pathogenic and an avirulent strain of *E. stewartii*, respectively, thus eliminating them as cross-reactive strains. Additional serological testing of the other possible cross-reactive strain, P-53, showed that when culture medium, used to support the growth of SP2/O cells (containing no specific antibodies), was substituted for the culture medium containing the monoclonal antibody, the absorbance was not affected (data not shown). This indicates that the variable positive/negative monoclonal antibody based indirect ELISA response of strain P-53 was the result of an interaction between the P-53 bacterial strain and the enzyme-labeled antimouse IgG antibody and not between the specific monoclonal antibody and strain P-53. Therefore, a control well should be included in which culture medium from SP2/O cells is substituted for the culture media containing the specific antibody in an indirect ELISA for each bacterial sample.

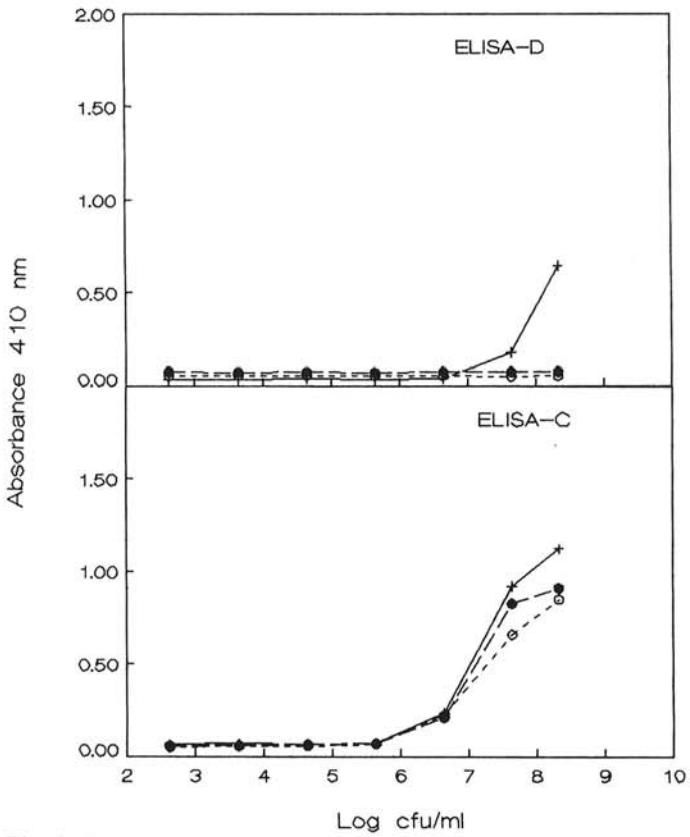

Fig. 2. Absorbance profiles of enzyme-linked immunosorbent assays (ELISAs) C and D for detection of *Erwinia stewartii* in phospate-buffered saline (PBS) alone and in PBS amended with corn seed supernatants A632 or Hybrid Pride of Canada (HPC). ELISA-D was an indirect ELISA using the specific monoclonal antibody C/G7/B2 (1:50 dilution of hybridoma culture medium) and an alkaline phosphatase-conjugated antimouse immunoglobulin (1:1,000). When no bacteria were added to the sodium carbonate buffer, sodium carbonate plus A632 supernatant, and sodium carbonate plus HPC supernatant, the absorbance means were 0.032, 0.052, and 0.077, respectively. Polyclonal capture antibody (10 μg/ml), monoclonal second antibody (10 μg/ml), and alkaline phosphatase-conjugated antimouse immunoglobulin (1:1,000) was used in ELISA-C. When no bacteria were added to the PBS, PBS plus A632 supernatant, and PBS plus HPC supernatant, the absorbance means were 0.052, 0.052, and 0.056, respectively. Data represent PBS (ELISA-C) or sodium carbonate (ELISA-D) diluent (+——+), PBS or sodium carbonate diluent containing A632 seed supematant, respectively (O- - -O), and PBS or sodium carbonate diluent containing HPC seed supernatant, respectively (● — — ●).

Extensive screening of bacterial strains indicated that the monoclonal antibody recognized, and was specific to, a wide range of virulent *E. stewartii* strains but that it did not distinguish between virulent and avirulent strains of *E. stewartii*. Although it did react with three known avirulent strains of the pathogen, it is possible that unidentified bacteria from corn seeds or plants and not detected by the monoclonal antibody were avirulent *E. stewartii* strains.

Comparison of the double-sandwich assays, ELISA-A, ELISA-B, and ELISA-C revealed they all detected viable *E. stewartii* bacteria in the presence of corn-seed supernatant. ELISA-D, however, which was not a double-sandwich ELISA, did not do so, eliminating it for consideration as a seed assay. This may have been due to the seed tissue competing for and/or physically blocking the binding sites for the bacteria in the wells of the microtiter plates. Although ELISA-A had low detection limits, it also was not suitable for a seed assay because of the lack of specificity of the polyclonal antibodies. ELISA-B and ELISA-C used the monoclonal antibody C/G7/B2, which was highly specific for virulent, *E. stewartii*. ELISA-C, however, had detection limits 6.7, 2.5, and 1.7 times lower than ELISA-B for *E. stewartii* in PBS, in PBS containing A632 seed supernatant, and in PBS containing HPC supernatant, respectively. This may be explained by previous observations suggesting that monoclonal antibodies often do not work as well as polyclonal antibodies for capture and that conjugation of the monoclonal antibody to alkaline phosphatase may sterically interfere with binding to the antigen or cause conformational changes in the antibody, thus altering its affinity or specificity for the antigen (10,17). ELISA-C is the best choice for use in a seed assay because it is highly specific, has a low detection limit for *E. stewartii* in the presence of corn-seed tissue, and does not require the conjugation of alkaline phosphatase to the antibodies.

The strongest response of ELISA-A was to the ground and to the supernatant fraction of seed preparations of A632 seeds from plants inoculated with strain SS104. This finding concurs

TABLE 5. Absorbance values obtained by enzyme-linked immunosorbent assay (ELISA)[a] of corn seed harvested from Hybrid Pride of Canada corn plants either inoculated with *Erwinia stewartii* or not inoculated

Sample tested	Source	Mean[b]	SD
Corn seed[c]	Inoculated plants	0.338	0.089
Corn seed	Uninoculated plants	0.056	0.014
9A[d]	Control culture	1.014	0.304
SS104[e]	Control culture	1.104	0.227
A.T.[f]	Control culture	0.068	0.013
PBS[g]		0.072	0.023

[a] The ELISA (ELISA-C) with polyclonal capture antibodies (10 μg/ml), monoclonal antibody C/G7/B2 (10 μg/ml) as the second antibody, and an alkaline phosphatase-labeled antimouse immunoglobulin (1:1,000) was used.
[b] Mean value of four wells per plate replicated on three plates.
[c] Samples were derived from seed (40 g) of Hybrid Pride of Canada sweet corn grown in the greenhouse, either inoculated with *E. stewartii*, strain 9A, or uninoculated.
[d] A rif^r and nal^r mutant isolated from *E. stewartii*, strain DC283 (nal^r).
[e] The immunizing *E. stewartii* strain for production of the polyclonal and monoclonal antibodies used in the ELISA.
[f] Negative control bacterium, *Agrobacterium tumefaciens*.
[g] Phosphate-buffered saline (0.02 M Na_2PO_4, 0.85% NaCl, pH 7.2).

with other research that suggests the bacterium is internally borne in the seed (8). Seed samples should therefore be ground and clarified to obtain the optimum ELISA response.

The ELISA-C readily distinguished between bulk sweet-corn seed samples from HPC plants inoculated with strain 9A or uninoculated. These results were confirmed by recovery and positive identification of strain 9A isolated from the seed lot harvested from inoculated plants. Strain 9A, used in these studies, had physiological and biochemical properties similar to other *E. stewartii* strains and produced typical Stewart's wilt symptoms when inoculated into sweet corn or dent corn. Therefore, these results should be representative of testing for wild-type strains of *E. stewartii* in corn seed.

There was an absolute correlation between a positive ELISA-C absorbance value greater than 0.40 and the recovery of strain 9A from individual seeds. The ELISA-C did not distinguish between 10 seeds with absorbance values below 0.40 from which *E. stewartii* was recovered and seeds from which no bacteria were recovered. The detection limit for ELISA-C in this experiment, however, would be the equivalent of recovering approximately 10^5 colonies per plate. Nine of the 10 seeds were well under this threshold, and the other seed may also have been. The low number of bacteria recovered from these nine individual seeds may be the result of low numbers of bacteria present in a particular seed, a seed not sufficiently fractured to release many bacteria, or a seed in which many of the bacteria were not viable. Low absorbance values were associated with the recovery of low numbers of bacteria. This suggests that there were not large numbers of nonviable bacteria present because the ELISA does not distinguish between viable and nonviable bacteria. As discussed previously, ELISA-C did readily distinguish between the bulk seed lots from which these individual seeds came. The bulk samples were finely ground. The grinding process probably releases a much larger percentage of the bacteria from the infected seeds than does

TABLE 6. Results of identification tests of bacterial isolates obtained from corn seed harvested from diseased Hybrid Pride of Canada plants grown in the greenhouse

Bacterial isolates[a]	Origin	Media[b]	Diagnostic tests				
			Gram stain[c]	Motility[d]	EPS[e]	Pathogenicity[f]	Absorbance[g]
1	9A inoc.	rif/nal	−	−	+	+	0.457
2	9A inoc	rif/nal	−	−	+	+	0.431
3	9A inoc.	nal	−	−	+	+	0.311
4	9A inoc.	nal	−	−	+	+	0.394
SS104	Culture	None	−	−	+	+	0.309
DC283	Culture	None	−	−	+	+	0.754
9A	Culture	None	−	−	+	+	0.572
A.T.	Culture	None	ND[h]	ND	ND	ND	0.030

[a] Isolates 1–4 were recovered from seed harvested from Hybrid Pride of Canada sweet corn inoculated with strain 9A. The positive controls were *Erwinia stewartii* strains SS104, DC 283, and 9A. The negative control was *Agrobacterium tumefaciens* (A.T.).
[b] Rifampicin was added at 50 mg/L and nalidixic acid at 25 mg/L to nutrient agar containing 50 mg/L of cycloheximide and 0.05% antifoam B.
[c] + = Positive, − = negative.
[d] Determined by observing a hanging drop of bacterial suspension with a compound microscope. + = Positive, − = negative.
[e] Bacterial isolates were grown on nutrient agar containing 1% dextrose and were observed for production of extracellular polysaccharide. + = Positive, − = negative.
[f] Pathogenicity was determined by inoculation of corn seedlings with the bacterial isolates. Inoculated seedlings were observed for 2 wk for development of characteristic disease symptoms. + = Positive, − = negative.
[g] Absorbance at 410 nm from an indirect ELISA with monoclonal antibody C/G7/B2 (1:50 dilution of hybridoma culture medium) and an alkaline phosphatase-labeled antimouse immunoglobulin (1:1,000) (ELISA-D).
[h] Not determined.

TABLE 7. Comparison of enzyme-linked immunosorbent assay (ELISA)[a] absorbance values and recovery of *Erwinia stewartii* from individual corn seeds

Absorbance range[c]	Seed lots[b]							
	A632				HPC			
	Uninoculated		Inoculated		Uninoculated		Inoculated	
	No. of seeds	Isolates recovered[d]	No. of seeds	Isolates recovered	No. of seeds	Isolates recovered	No. of seeds	Isolates recovered
0.0–0.199	16	0	195	6	15	0	191	3
0.2–0.399	0	...	0	...	1	0	6	1
0.4–0.599	0	...	1	1	0	...	0	...
0.6–0.799	0	...	1	1	0	...	1	1
0.8–0.999	0	...	0	...	0	...	0	...
1.0–1.199	0	...	1	1	0	...	0	...
1.2–1.399	0	...	1	1	0	...	0	...
1.4–1.599	0	...	0	...	0	...	1	1
1.6–1.799	0	...	1	1	0	...	1	1
1.8–1.999	0	...	0	...	0	...	0	...
Totals	16	0	200	11	16	0	200	7

[a] The double-sandwich ELISA (ELISA-C) with polyclonal antibodies (10 μg/ml) as capture antibodies, monoclonal antibody C/G7 B2 (10 μg/ml) as the second antibody, and an alkaline phosphatase-labeled antimouse immunoglobulin (1:1,000) as the signal antiody was used.
[b] 'Diseased seeds' were harvested from plants of corn inbred A632 grown in the field in 1988 and inoculated with *E. stewartii* strain 9A (rif^r, nal^r). 'Healthy seeds' were from uninoculated plants. Hybrid Pride of Canada (HPC) plants were grown in the greenhouse in 1988, and seeds were harvested from either inoculated or uninoculated plants.
[c] Absorbance measured at 410 nm. Values above the first two rows were regarded as negative by ELISA.
[d] Number of seeds from which bacterial isolates were recovered on nutrient agar amended with 50 mg/L rifampicin, 25 mg/L nalidixic acid, 50 mg/L cycloheximide, and 0.05% antifoam B.

fracturing. Therefore, adequate grinding of the sample to release all bacteria into the sample buffer is critical to a successful seed assay for *E. stewartii*.

The average number of *E. stewartii* in an infected seed is unknown, as is the average number of infected seeds coming from infected plants. In this study, bacteria were recovered from 5.5 and 3.5% of the A632 seeds from the field and the HPC seeds from the greenhouse, respectively. Further studies are necessary to determine the average number of *E. stewartii* in an infected seed and the average number of infected seeds present in infected plants. With this information and with the data we have developed on detection limits, the probability of detecting specific numbers of infected seeds in different sample sizes can be determined, as can the appropriate sample size for detecting seedborne *E. stewartii* by ELISA-C.

The ELISA-C procedure uses a highly specific monoclonal antibody and has a low detection limit, and positive ELISA responses from bulk and individual seed samples have been correlated with recovery of *E. stewartii*. The procedure can thus be reliably used as the basis for serological testing for seedborne *E. stewartii* in corn seed.

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