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Comparison of immunofluorescence colony-staining in media, selective isolation on pectate medium, ELISA and immunofluorescence cell staining for detection of Erwinia carotovora subsp. atroseptica and E. chrysanthemi in cattle manure slurry

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Abstract

Various isolation and serological techniques were compared for the detection of *Erwinia carotovora* subsp. *atroseptica* (Eca) and *E. chrysanthemi* (Ech) in cattle manure slurry containing c. 10^8 colony forming units (cfu) per ml. The slurry samples could be preserved at −80 °C for 8 months without reduction in the number of bacteria but not at −20 °C. Samples stored at −80 °C were inoculated with concentrations of the target bacterium ranging from 10^2 to 10^8 per ml. Only immunofluorescence colony-staining (IFC) in combination with selective media was able to detect the target organism at a concentration of 100 cells per ml. No IFC-positive colonies were found in pour plates of the non-inoculated cattle slurry. The recovery of the target bacterium from slurry inoculated with 10^2 cfu of Ech per ml was 64% in PT medium (containing polygalacturonic acid) and 19% in crystal violet pectate medium (CVP). Recoveries of Eca were 32% and 82%, respectively. Ech and Eca could be detected at levels of 10^3 cfu per ml of slurry by isolation on CVP. Crude filtration procedures were necessary for analysis of slurry samples with immunosorbent immunofluorescence (ISIF) cell staining. The detection level of ISIF for Ech was 10^5 cells per ml of slurry. IF-positive cells were incidentally observed in the non-inoculated slurry. Detection of Ech and Eca with ELISA was only possible in slurry inoculated with 10^8 cells of the target bacterium per ml.

Additional keywords: sample preservation, sample filtration, detection level, total counts.

Introduction

In the Netherlands, high-grade seed potatoes are certified for *Erwinia carotovora* subsp. *atroseptica* (Eca) and *E. chrysanthemi* (Ech), and there is a zero-tolerance level for diseases caused by these two pathogens in motherplants in the field. In addition tubers are tested for detection of Eca after harvest with ELISA in the laboratory (Van Vuurde

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et al., 1988). Regional excess of cattle slurry from dairy farms is applied to fields on seed potato farms. Concern has arisen about the risk of introduction of pathogenic *Erwinia* spp. into disease-free farm land. Therefore, a reliable system capable of detecting low levels of these pathogens in cattle slurry is needed to assess this risk.

Plating methods on a semi-selective medium, e.g. crystal violet pectate medium, CVP (Cuppels and Kelman, 1974) enable the isolation of the target bacterium and offer the possibility of a positive identification of the isolated strain. However, plating procedures are less suitable for quantitative population dynamics studies, as Ech or Eca cannot be distinguished from other *Erwinia* spp. or subsp. on the dilution plates. High-quality antisera are available now for serological characterization of the only known Dutch serotype of Eca or Ech from potato and to distinguish these serotypes from the Dutch *E.c.* subsp. *carotovora* (Ecc) serotypes and over 99% of other bacteria isolated from potato peel extract (J.M. van der Wolf, 1990).

Therefore, the detection of Eca and Ech in cattle slurry was studied with enzyme-linked immunosorbent assay (ELISA), immunofluorescence microscopy (IF), immunosorbent IF (ISIF) and immunofluorescence colony-staining (IFC). ELISA and IF were chosen due to their suitability for routine screening of large numbers of samples. In ISIF, the target bacteria are selectively trapped from the sample, allowing subsequent removal of most of the non-bound particles and bacteria by gentle washing (Van Vuurde and Van Henten, 1983). Compared with IF this results in less interference of slurry particles when observing stained cells in the preparation. The potential of IFC (Van Vuurde, 1987) for detection of Eca and Ech in population dynamics studies was investigated. The advantage of IFC for our study is that it combines isolation of the viable target organism with a direct serological differentiation between the target colony and saprophyte colonies in the agar medium. To enable comparison of tests performed over a period of eight months cattle slurry was preservated by freezing. The validity of this preservation method was previously evaluated under various conditions.

For practical reasons the experiments were focused on Ech detection as Ech is considered the most dangerous *Erwinia* spp. for seed potato production, but most procedures were also tested for detection of Eca.

Materials and methods

Bacterial strains. For Ech we used the strains: IPO (Research Institute for Plant Protection) no. 502 (reference strain, used in all experiments with Ech), 775, 777, 824 (= SCRI 4011) and for Eca: IPO no. 161. Ech strains were grown on a growth-factors medium containing per l: 0.4 g K_2HPO_4, 0.05 g $MgSO_4.7H_2O$, 0.1 g NaCl, 0.5 g $NH_4H_2PO_4$, 0.01 g $FeCl_3$, 1 g glucose, 3 g yeast, 15 g agar. The Eca strain was grown on a broth medium containing per l: 8 g Lab-Lemco broth, 5 g NaCl, 15 g agar. Slope cultures of the strains were grown for 24 h at 27 °C. Dilutions of the cultures were prepared in quarter strength Ringer's solution (Becton and Dickenson, 1985) for isolation and IFC experiments or in 0.01 M phosphate buffered saline, PBS, pH 7.4 (Clark and Adams, 1977), with 0.1% Tween 20 (PBS-T) for ELISA, IF and ISIF experiments.

Cattle slurry. The cattle slurry was obtained from the experimental farm 'De Ossekampen' in Wageningen. It had a dry matter content of 8.3% and a pH of 7.6. Samples were stored at −80 °C to maintain as much similarity as possible for the experiments

with the various detection methods. Changes in the number of colony forming units (cfu) of the indigenous bacteria were determined in the frozen slurry over a period of time. Replicate subsamples of 50 ml each in plastic bags were placed at −20 °C and at −80 °C. For the samples stored at −80 °C, the effect on the number of cfu following the addition of 0.05 g $CaCl_2$ per l of cattle slurry and that of slow and fast freezing was also studied. Slow freezing was done by placing the bags with the cattle slurry in a 1.2 l polystyrene box with inner measurements of 24.4 × 11.3 × 4.4 cm (l × w × h) and 2.0 cm thick walls. Fast freezing was done in plastic bags without the polystyrene box. Directly before inoculation, the cattle slurry was thawed by incubation in a water-bath at 40 °C for 5 to 10 min. The number of cfu was determined for the various treatments and storage intervals by agar-mixed (pour) plating of sample dilutions with trypticase soy agar (TSA, BBL no. 11043).

In the various experiments, the thawed cattle slurry was inoculated with suspensions of Eca or Ech strains or dilution buffer to 10% of the final volume of the slurry test sample. The pathogens were not longer than one hour exposed to the cattle slurry before isolation.

Isolation media and plating procedures. TSA was used as general plating medium for *Erwinia* strains and for saprophytic microorganisms. CVP and the polygalacturonic acid containing PT medium (Burr and Schroth, 1977) were used for semi-selective isolation of *Erwinia* spp. Purified agar was replaced by technical agar (Difco no. L13) and Tergitol by Tween 20 for the preparation of PT.

Inoculum concentrations for the various dilutions of the Ech and Eca strains and the slurry test samples were determined by surface plating of 100 µl of a dilution series in quarter-strength Ringer's solution on CVP.

Plating efficiency of Ech and Eca in PT, CVP and TSA were determined by pour plating of 50 or 100 µl of a dilution of the test strain with 5 ml of liquid (45 °C) agar medium in a 5 cm diameter Petri dish. Plate counts of the test strain showed that it contained between 10^3 to 10^4 cfu per ml. Inoculated plates were incubated for 48 h at 27 °C. Problems with solidification of CVP at 45 °C before mixing with the sample were solved by adding 5 g sodium citrate per l before autoclaving.

Pour plates for IFC were prepared as described in the paragraph on the IFC-method.

Antisera and conjugates. The anti-Ech and anti-Eca sera were prepared at IPO by injecting a suspension of washed viable bacteria into a rabbit (Vruggink and Maas Geesteranus, 1975). The fluorescein isothiocyanate (FITC) – conjugates were prepared according to the method of Allen and Kelman (1977) and the alkaline-phosphatase conjugates according to that of Tóbiás et al. (1982). The various antisera, antiserum conjugates, antiserum dilutions and test conditions used for the serological tests are summarized in Table 1. Optimal antiserum dilutions were determined by titration.

Immunofluorescence microscopy. Preparations of IF, ISIF and IFC experiments were observed with a Leitz Orthoplan microscope with an internal tube-magnification factor of 1.25 and equiped with incident blue light and filter system for FITC (Leitz I_2). Bacteria trapped and stained on the bottom of 96-well microtiter plates with ISIF, were observed through the bottom of the inverted plate with a 40× long-distance objective (Olympus LWDCDPL/NA 0.55; with 1.9 mm working distance and coverglass correc-

Table 1. Scheme of antiserum preparations, antiserum dilutions, dilution buffers and incubation conditions used in the serological tests.

Application	Preparation of serum	Target bacterium	IPO-code serum	Dilution factor	Dilution buffer	Incubation time (h)	Incubation temp. (°C)
Coating of IgG in ISIF	crude serum	–[1]	8575A1	100	CB[2]	18	6
		Ech	8575E1	100	CB	18	6
Staining in IFC	FITC-conjugated IgG	Ech	8174/5	100	PBS[3]	18	27
		Eca	8634C5	75	PBS	18	27
Staining in IF and ISIF	FITC-conjugated IgG	Ech	8174/5	100	PBS	0.5	27
Coating of IgG in ELISA	1 mg IgG ml^{-1} serum	Ech	8575E4	2000	CB	18	6
		Eca	8567G4	4000	CB	18	6
Enyme binding in ELISA	alkaline-phosphatase conjugated IgG	Ech	8575E6	2000	PBS-T[4]	5	27
		Eca	8567G6	4000	PBS-T	5	27

[1] Pre-imune serum.
[2] CB: carbonate buffer (50 mM CO_3^{2-}).
[3] PBS: phosphate bufferd saline (10 mM PO_4^{3-}).
[4] PBS-T: PBS + 0.1% Tween 20.

tion). Preparations of agar films stained for IFC were examined with a 4× objective (Leitz achromat, NA 0.10) and 4× ocular (Leitz, Periplan GW). Transmitted light with or without phase-contrast illumination was used to examine preparations for IF-negative cells or colonies of bacteria.

Immunofluorescence colony-staining (IFC). Centrifugation and crude filtration were used to increase the concentration of bacteria and to reduce the interference of sample particles in the slurry preparation for IF and IFC. For each test sample a filter consisting of a double layer of cheese cloth with cotton in between was mounted in a 250 ml centrifuge tube. A 10 ml sample of undiluted slurry was loaded on the filter and crudely filtered and centrifuged through the filter at 8000 *g* for 15 min, thus concentrating the bacteria and other fine particles in the pellet. The pellet was resuspended in 4 ml of quarter-strenght Ringer's solution.

Pour-plates of the samples were prepared with various isolation media for *Erwinia* spp. Differentiation between the colonies of the target bacterium and those of saprophytic microorganisms was done by staining the colonies with FITC-conjugated antiserum (Van Vuurde, 1987). Gels were prepared by mixing 25 µl of a crudely filtered and centrifuged slurry suspension with 0.5 ml of agar medium at 45 °C in 16 mm diameter wells of a 24-well tissue-culture plate (Costar no. 3424).

After incubation the medium was dried in the well into a thin agar film with a warm air blower at c. 40 °C. The target colonies were stained by incubating the agar film in the well for 18 h with 300 µl of diluted FITC-conjugated antiserum (see Table 1 for incubation conditions). The agar film was incubated twice for 10 min each with 1 ml of 0.001 M PBS to wash away unbound conjugate. The stained agar films were examin-

ed directly in the wells of the plate with a fluorescence microscope at a 20 times magnification. Simultaneously prepared agar films from the filtered and centrifuged cattle slurry non-inoculated with target bacteria were used as controls on cross reactions in four different experiments. Agar films with dilutions of pure cultures of the target bacterium served as positive controls.

Immunosorbent IF (ISIF). The following procedure was designed for handling of test samples in ISIF. Wells of a flatbottom 96-well microplate were coated with 150 μl of diluted antiserum according to the scheme in Table 1, and washed twice for 5 min with 150 μl of PBS-T. The experiment was performed with cattle slurry containing 10^7 Ech cells per ml. For crude clearing of the cattle slurry, a filter system was made in 1 ml disposable pipet tips which had 5 mm of the tip cut off and which were tightly filled with cotton. The tips were placed in a polystyrene template and mounted above the antibody-coated wells of a microtiter plate (Fig. 1). Each filter system was loaded with 0.5 ml of cattle slurry. The plate with the filter sytems was centrifuged for 15 min at 440 *g* using a swing-out rotor for microplates (Heraeus-Christ Minifuge GL, type 4400). The filter systems were removed after centrifugation and the wells were gently washed with PBS. The period of PBS incubation and the number of washes were varied to investigate the effect of the washing procedure on the specificity of immunotrapping. Trapped cells were fixed onto the bottom of the well by applying 96% ethanol for 10 min and stained for IF directly in the well. Non-inoculated cattle slurry and a dilution of the pure culture of the target bacterium were used as controls.

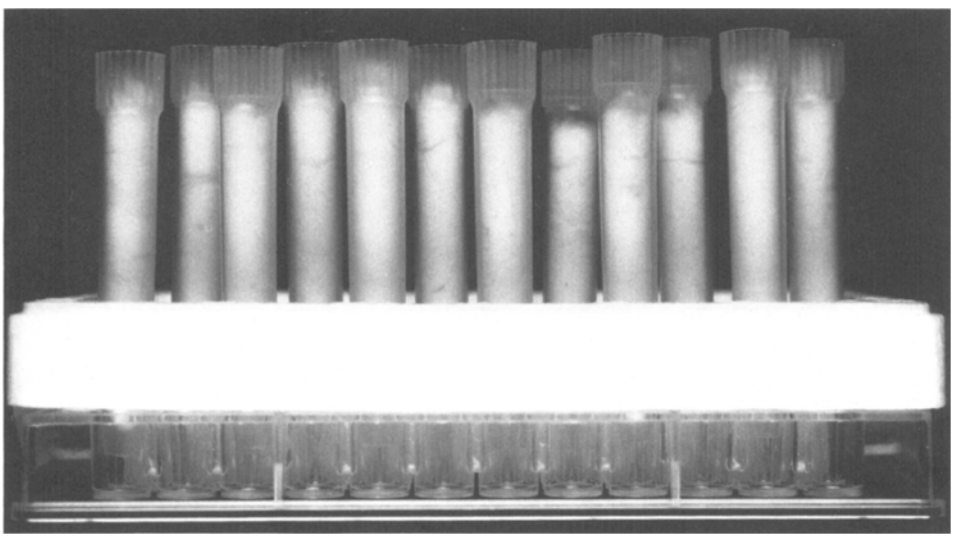

Fig. 1. Filter system using a 96 well microplate, a styropore template and 1 ml pipet tips for crude purification of cattle slurry samples for immunosorbent IF. The pipet tips are filled with cotton. Half a ml of crude cattle slurry is filtered through the coton in a pipet tip by centrifuging the whole system in a swing-out rotor at 440 *g* for 15 min.

ELISA. The detectability of Eca and Ech with the double antibody sandwich ELISA (DAS-ELISA) was determined for cattle-slurry samples inoculated at various concentrations and for ten-fold dilution series of pure cultures of Eca and Ech. DAS-ELISA was performed as described by Clark and Adams (1977) with the slight modifications presented in the scheme of Table 1. The alkaline-phosphatase-conjugated antibodies were prepared according to Tobias et al. (1982). Washings were done vigorously with tap water using a perspex apparatus for simultaneous washing of 96 wells. The absorbance values were measured at 405 nm and 690 nm (reference for compensating non-specific absorption from the well) with a photometer (SLT Easy Reader EAR 400) after 15, 30, 60 and 120 min of incubation with the enzyme.

Statistical analysis. Analysis of variance and tests for least significant difference were used for comparison of data.

Results

Preservation of microorganisms in frozen cattle slurry. The effects of various storage conditions and periods on the survival of bacteria in non-inoculated cattle slurry are presented in Table 2A and 2B. The concentration of viable bacteria in cattle slurry frozen at −20 °C decreased 80% in the first 29 days and remained constant for 200 days thereafter (Table 2A), whereas concentrations in samples stored at −80 °C (Table 2B) remained constant for at least 8 months.

Addition of $CaCl_2$ to the cattle slurry before freezing and slow or fast freezing of the sample to −80 °C did not significantly affect ($P = 0.05$) the total number of cfu over the test period. The average total number of cfu for the samples stored at −80 °C varied on the various sampling dates from 0.4 to 1.4 times the number before freezing. This variation resulted in somewhat higher plate counts for all sample treatments at day 35 (not significant at $P = 0.05$) and significantly lower ($P = 0.05$) at day 101, compared with the plate counts before freezing. The samples stored at −80 °C were used in further experiments on comparison of methods.

Detection on CVP. Preliminary experiments with undiluted cattle slurry showed that less than 10 µl of an undiluted sample could be plated on CVP to allow Ech cells to form the cup-like depressions typical for *Erwinia* spp. The detectability of Ech and Eca in cattle slurry by surface plating on CVP was better than 1000 cfu per ml (Table 3). No typical cup-like depressions on CVP were found for dilution series of non-inoculated cattle slurry. The recovery from slurry on CVP was between 34% and 114% for Ech strains, and for Eca between 88% and 152% of the number obtained with plating the pure culture dilution of the inoculum on TSA.

Immunofluorescence colony-staining. The plating efficiency of various strains of Ech and Eca in pour plates with PT, CVP and TSA were compared to select a suitable medium for IFC. The plate counts were only significantly lower ($P = 0.05$) for Ech strain IPO 777 in PT compared with TSA (Table 4). The plating efficiencies of the Ech strains varied between 83 and 112% for CVP and 79 and 112% for PT as compared with those for TSA.

The percent recovery in IFC from cattle slurry inoculated with various concentra-

Table 2A. Plate counts for cattle slurry sample with (8.0 ± 1.1) × 10^7 cfu per ml after various periods of storage at −20 °C.

Days of frozen storage	Cfu (× 10^7 ml^{-1} slurry)
0	8.0 ± 1.1
7	8.4 ± 0.3
22	3.4 ± 0.5
29	1.5 ± 0.7
88	1.6 ± 0.3
134	1.5 ± 0.5
197	1.5 ± 0.3
239	2.6 ± 0.6

Table 2B. Plate counts for cattle slurry samples with (10.7 ± 1.4) × 10^7 cfu per ml after various periods of storage at −80 °C. Samples were stored with or without $CaCl_2$. Freezing was done fast without polystyrene isolation around the sample (−PS) or slowly with PS. Data represent mean and standard deviation of 5 replicates.

Storage period (days)	Cfu (× 10^7 ml^{-1} slurry)			
	No $CaCl_2$ added		$CaCl_2$ (0.05 g l^{-1}) added	
	− PS	+ PS	− PS	+ PS
10	7.2 ± 3.9	4.9 ± 1.6	5.0 ± 2.7	5.7 ± 6.1
35	11.9 ± 3.6	11.1 ± 3.1	25.7 ± 20.8	11.4 ± 7.2
67	6.6 ± 2.0	8.2 ± 0.9	7.3 ± 1.4	4.9 ± 1.3
101	4.1 ± 0.8	4.4 ± 0.5	3.1 ± 0.9	5.4 ± 1.4
147	8.3 ± 1.7	8.5 ± 2.2	8.8 ± 1.7	7.9 ± 1.0
210	14.6 ± 2.9			
252	12.6 ± 2.4			

tions of Ech or Eca was significantly higher (P = 0.05) for the semi-selective media PT and CVP than from TSA (Table 5A, B). The non-selective TSA plates often showed extremely high numbers of saprophytes, strongly reducing the detectability of Eca and Ech. The percentage recoveries of Ech and Eca for PT were about 2 times higher than for CVP. The recoveries of Eca were 10-60% higher than those of Ech in PT and CVP. Ech and Eca could not be detected when 25 µl of slurry-extract was mixed with 0.5 ml of TSA.

The colonies of Eca and Ech in PT were relatively large and were stained bright green against a dark background with small colonies of indigenous cattle slurry bacteria and slurry particles (Fig. 2). In our experiment up to 400 Eca (Table 5B) colonies could be detected among over 1000 saprophyte colonies in 0.5 ml agar medium. Compared with PT, the staining in CVP was a slightly more brilliant for Eca and Ech and the pathogen colonies stayed smaller. However, the background fluorescence of CVP was

Table 3. Recovery of A) *Erwinia chrysanthemi* and B) *Erwinia carotovora* subsp. *atroseptica* from artificially inoculated slurry by surface plating of 100 μl of the different dilutions on CVP. Data represent mean and standard deviation of 5 replicates.

Inoculation concentration[1]	Dilution factor slurry	Pectinolytic colonies	Recovery (%)
A) *Erwinia chrysanthemi*			
1.1×10^3	10	8.2 ± 5.6	73
1.1×10^3	100	0.6 ± 0.9	54
1.1×10^4	10	37.0 ± 16.4	34
1.1×10^4	100	12.6 ± 4.7	114
1.1×10^5	10	confluent	
1.1×10^5	100	72.6 ± 17.5	66
0.0	10	0.0 ± 0.0	
0.0	100	0.0 ± 0.0	
B) *Erwinia carotovora* subsp. *atroseptica*			
5.0×10^2	10	7.6 ± 2.9	152
5.0×10^2	100	0.6 ± 0.9	120
5.0×10^3	10	43.8 ± 7.4	88
5.0×10^3	100	6.4 ± 1.8	128
5.0×10^4	10	confluent	
5.0×10^4	100	59.4 ± 11.9	118
0.0	10	0.0 ± 0.0	
0.0	100	0.0 ± 0.0	

[1] The inoculation concentration (cfu per ml slurry) is calculated from plate counts from dilutions of the pure cultures on TSA.

Table 4. Average plating efficiencies (4 replicates) for pour plating of various *Erwinia chrysanthemi* (Ech) strains and *Erwinia carotovora* subsp. *atroseptica* (Eca) strain 161 in crystal violet pectate (CVP) medium and PT compared with trypticase soy agar (100%). Values with dissimilar superscripts (horizontal comparison) are significantly different at $P = 0.05$.

Pathogen	IPO no. of strain	Plating efficiency (%)	
		CVP	PT
Ech	502	99[a]	95[a]
Ech	775	93[a]	94[a]
Ech	777	83[a]	79[b]
Ech	824	112[a]	112[a]
Eca	161	95[a]	102[a]

Table 5. Plate counts and percentage recovery of *Erwinia chrysanthemi* (A) and of *Erwinia carotovora* subsp. *atroseptica* (B) from artificially inoculated cattle slurry by immunofluorescence colony-staining (IFC) in PT, CVP supplemented with 5 g sodiumcitrate per liter and TSA. Data represent mean and standard deviation of positive colonies per well (ϕ = 16 mm) in tissue culture plates (5 replicates).

Inoculation concentration[1]	PT		CVP		TSA	
	IFC-positive colonies	recovery[2] (%)	IFC-positive colonies	recovery (%)	IFC-positive colonies	recovery (%)
A. *Erwinia chrysanthemi*						
1.0×10^2	4.0 ± 2.6	64	1.2 ± 1.0	19	0.0 ± 0.0	0
1.0×10^3	17.1 ± 4.1	27	8.7 ± 2.6	14	0.0 ± 0.0	0
1.0×10^4	147.8 ± 17.4	24	64.3 ± 29.0	10	0.0 ± 0.0	0
0.0	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	
B. *Erwinia carotovora* subsp. *atroseptica*						
9.0×10^1	4.6 ± 2.3	82	1.8 ± 0.4	32	0.0 ± 0.0	0
9.0×10^2	28.6 ± 13.3	51	15.8 ± 5.2	28	0.0 ± 0.0	0
9.0×10^3	371.8 ± 65.9	66	216.4 ± 8.7	38	0.0 ± 0.0	0
0.0	0.0 ± 0.0		0.0 ± 0.0		0.0 ± 0.0	

[1] The concentration was calculated from plate counts from dilutions of the pure culture pour plated in TSA.

[2] Recovery is number of positive colonies / (0.025 × 2.5 × inoculation concentration), 0.025 is the volume of the sample (ml), 2.5 is the factor of concentration of the sample by centrifugation and filtration.

greener and more variable than that of PT. In TSA many cattle slurry bacteria form large yellowish colonies, which may be confused with weakly stained Ech or Eca colonies. The detectability of Eca and Ech was better than 100 cells per ml cattle slurry in PT and CVP pour plates (Table 5A, B). Most of the superficial colonies were washed away during the incubation of antiserum and washing procedure. None of the thousands of colonies present in the negative control samples of the non-inoculated cattle slurry gave positive colonies with IFC. This could be repeated in 4 different experiments on IFC.

Reisolation of pectinolytic *Erwinia* spp. from IFC-positive colonies by puncturing the colony with a glass capillary tube followed by plating on CVP was successful for 3 out of 8 colonies from various samples. However, the number of saprophyte colonies per cm^2 agar was often more that 100, and cells from these colonies heavily contaminated the sampling from an IFC-positive colony, thus strongly reducing the chance on successful reisolation.

Immunosorbent immunofluorescence. Preliminary experiments for detecting Ech in cattle slurry smears on glass slides with traditional IF procedures showed that the slurry particles strongly interfered with the detection of IF-positive cells in the preparation. Using ISIF, it was possible to overcome this problem and to obtain good quality IF-stained preparations on the bottom of the anti-Ech serum coated well of a microplate.

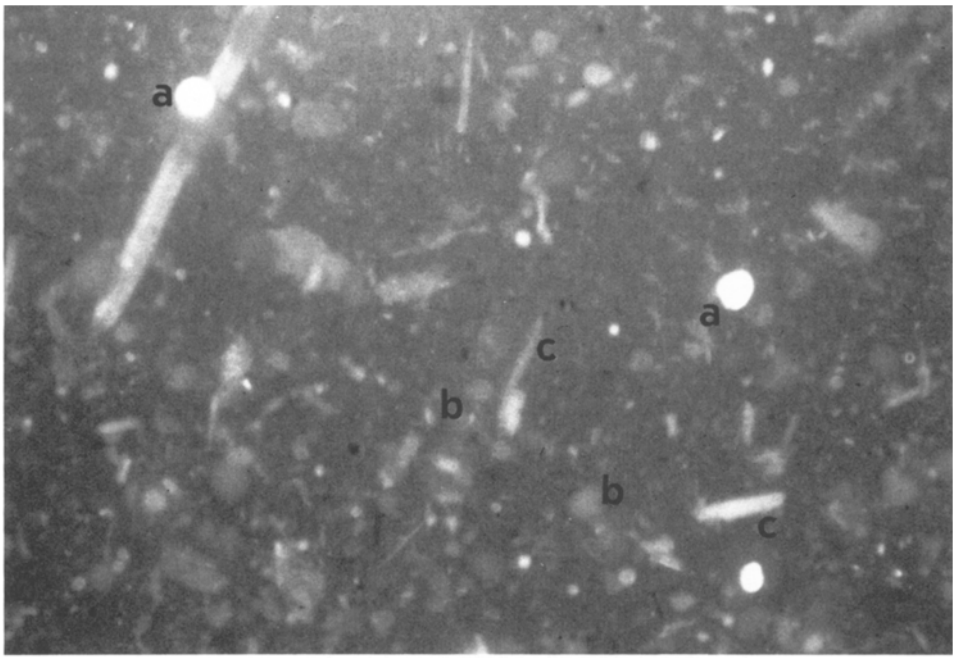

Fig. 2. Pour plate of diluted cattle slurry artificially inoculated with *Erwinia chrysanthemi* (Ech) and stained with immunofluorescence colony-staining after 48 h incubation at 27 °C. Brilliant (green) fluorescent colonies of Ech stained with FITC-labelled anti-Ech antibodies (a), among unstained colonies of saprophytes (b) and fine slurry particles giving a weak red autofluorescence (c). UV-microscope with objective magnification 4× and incident blue light.

The effect of the antibody coating and the washing procedure on the affinity and the specificity of the immunobinding is presented in Table 6. After incubation of the sample, different washing procedures were used: the wells were not washed, or incubated once or twice with PBS for 5 or 30 min. Trapping of IF-positive cells was 1.1 to 2.3 times higher in wells coated with specific serum than in wells coated with pre-immune serum. About 10% of the positive cells was washed away from the anti-Ech coating per wash during 5 min or 30 min washes. The number of positive cells in wells coated with pre-immune serum was not significantly changed by 5 min washes, but was significantly reduced ($P = 0.05$) c. 50% when the PBS buffer was incubated once or twice for 30 min. This procedure resulted for the various treatments in a detection level of c. 10^5 IF-positive bacteria per ml in cattle slurry inoculated with 10^7 Ech cells per ml. The negative control with non-inoculated cattle slurry showed a background of c. 1 IF-positive cell per microscope field.

Double antibody sandwich enzyme-linked immunosorbent assay. The detectability of Ech and Eca in suspensions of pure cultures with ELISA was between 10^5 to 10^6 per ml (Fig. 3). The background reaction in ELISA for cattle slurry was significantly lower for Ech as compared with Eca (absorbance values 0.14 and 0.52, respectively). Furthermore, the detectability of these pathogens in cattle slurry was significantly reduc-

Table 6. Effect of PBS-washes in ISIF on the number of cells of *Erwinia chrysanthemi* (Ech) from cattle slurry inoculated with Ech. Data represent the mean number and standard deviation of fluorescent cells per microscope field for 3 replicates.

Incubation time per wash (min)	Number of washes	Fluorescent cells per microscope field		Ss/Ps
		Specific serum (Ss)	Pre-immune serum (Ps)	
A. Cattle slurry inoculated with 10^7 Ech cfu per ml				
0	0	850 ± 225	575 ± 20	1.5
5	1	745 ± 150	580 ± 110	1.3
5	2	590 ± 185	555 ± 75	1.1
30	1	665 ± 45	285 ± 50	2.3
30	2	585 ± 145	265 ± 35	2.2
B. Non-inoculated cattle slurry				
5	2	1.0 ± 0.7	0.6 ± 0.6	1.7

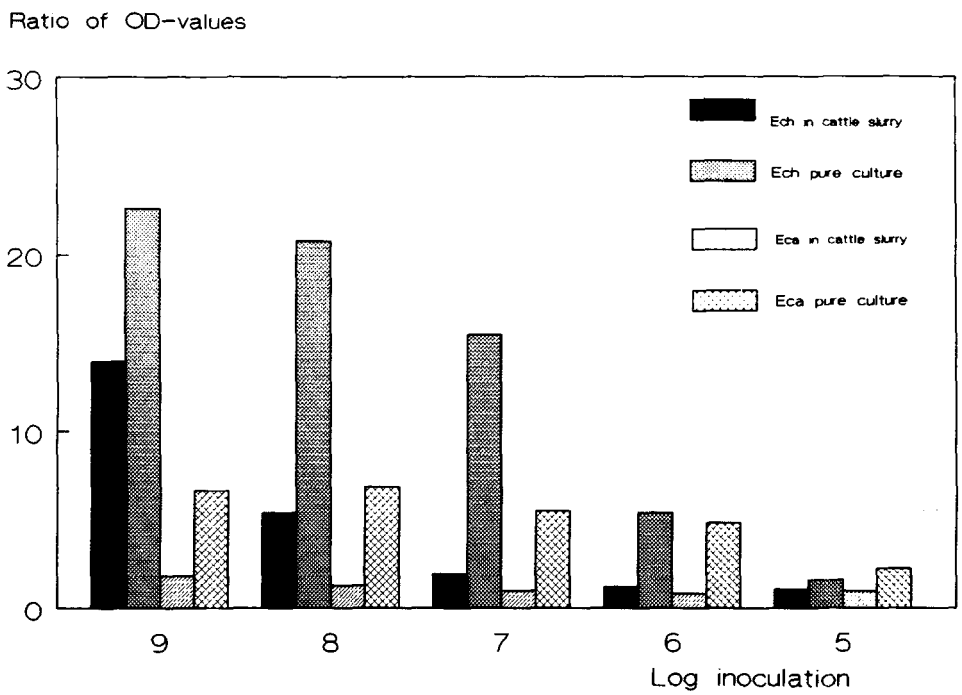

Fig. 3. Ratio of the ELISA absorbance value of cattle slurry inoculated with *Erwinia chrysanthemi* (Ech) or *E. carotovora* subsp. *atroseptica* (Eca) and the absorbance value of non-inoculated cattle slurry. As control on the interference of cattle slurry, the ratio of absorbance values was determined between PBS inoculated and non-inoculated with Ech or Eca.

ed as compared with the detection of the pure culture in suspension. At equal Eca or Ech concentrations the absorbance values were up to 6.25 times lower for cattle slurry than for suspensions of the pure cultures. The absorbance values of cattle slurry inoculated with 10^8 and 10^9 Ech or 10^9 Eca cfu per ml were significantly higher than the absorbance values of the back ground ($P = 0.05$).

Discussion

Preservation of samples for comparative testing. Experiments showed that storage at −80 °C, in contrast with −20 °C, did not result in changes in the total number of indigenous bacteria. This may be explained by the exposure of the bacteria to concentrated solutes at −20 °C, whereas all the water is frozen at −80 °C (Ingram and Mackey, 1976). Whereas Calcott (1978) reported that a cold shock injury to susceptible cells may be prevented by addition of calcium ions in the chilling fluid, we did not find an effect of $CaCl_2$ on viability. Significantly lower numbers were counted for all the experimental groups on one of the sampling days. This might be explained from non-optimal defrosting (Ingram and Mackey, 1976).

Specificity of the tests. According to Sheppard et al. (1986) the main conditions for tests for the detection of target organisms are a high diagnostic specificity (no false positive results) and a high diagnostic sensitivity (no false negative results). The risk on false positive results can be minimized when the detection assay is based on several characteristics of the pathogen. The distinction between viable and dead cells is important in research on the survival of Eca and Ech in cattle slurry. Viable cells are detected by isolation on CVP and by IFC. IF, ISIF and ELISA do not distinguish between dead and viable cells.

The detection and isolation of *Erwinia* spp. on CVP is based on the ability of the organism to hydrolyze polypectate and form typical cup-shaped pits in the medium (Cuppels and Kelman, 1974). No *Erwinia*-type pits were found on plates with 10-fold dilutions of non-inoculated cattle slurry. However, Eca, Ech and Ecc form identical pits and cannot be distinguished from each other on this medium. Incubation at differential temperatures (Pérombelon and Hyman, 1986) could not distinguish Dutch Ech strains from potato from various Ecc strains from potato (Janse and Spit, 1989).

Detection with IF and ISIF is based on both cell morphology and reaction with the FITC-conjugated antiserum. The coating antibodies in ISIF are directed against the target bacteria and were from a different antiserum as the antibodies used for fluorochrome conjugation. This may contribute to increase the specificity of the test compared with IF (Van Vuurde and Van Henten, 1983). However, both in IF and immunosorbent IF, some IF-positive cells were detected in the non-inoculated cattle slurry test samples at a density of 10^3 to 10^4 cells per ml. Against this background level, the detection level of Ech in our slurry sample can be estimated at c. 10^5 cells per ml. The relatively high non-specific binding of cells in wells coated with pre-immune serum in ISIF may be due to the centrifugation that forces the cells on the bottom of the plate and to the presence of antibodies against Ech in the serum. This pre-immune serum gave slight positive results in DAS-ELISA and in ELISA on nitrocellulose membranes (Van der Wolf, personal communication), possibly due to the development of antibodies against *Enterobacteriaceae* before immunization of the rabbit for antiserum produc-

tion. The lower binding strength of Ech-cell antigen for pre-immune serum antibodies in comparison with anti-Ech antibodies is demonstrated by the c. 50% reduction in numbers of Ech cells in wells coated with pre-immune serum after one or two times 30 min incubation with the wash buffer.

IFC combines both cultural and serological characteristics of the pathogen. The cultural characteristics are the ability of the target bacterium to grow in a semi-selective or elective medium. In some situations it is also possible to differentiate between colonies of the target bacterium and those of other microorganisms in the pour plate due to differences in colony size, morphology and/or color, as was demonstrated for cattle slurry inoculated with various dilutions of a pure culture of *Clavibacter michiganensis* subsp. *sepedonicus* (N.J.M. Roozen, J.W.L. van Vuurde and J.F. Chauveau, unpublished). IFC proved more suitable and reliable than immunodiffusion with antiserum added simultaneously to the pour plate (Van Vuurde, 1987) for further serological characterization of the target colony in the agar. The FITC-conjugated antiserum clearly differentiates between Eca or Ech and the colonies of other microorganisms and the slurry particles. Furthermore, in a small scale experiment we could confirm the identity of fluorescent colonies as that of a pectolytic *Erwinia* spp. by reisolation from the IF-positive colony. This shows that bacterial cells in IF-positive colonies can survive the detection procedure. The strong contamination from surrounding colonies of saprophytes affected the rate of successful isolation of the target organism from an IF-positive colony. However, we consider optimizing this technique very important for its potential to confirm actual presence of the target organism or to isolate cross-reacting bacteria if present in the sample.

The absence of IF-positive colonies in the non-inoculated slurry test samples shows that it is very likely that the positive reaction in inoculated slurry of the same sample is a reaction with the target organism and is not due to cross reactions. Furthermore, the absence of IF-positive colonies indicates that the IF-positive cells found with the c. 1000 times less sensitive IF and ISIF were non-viable Ech or Eca cells or cells of cross-reacting microorganisms that do not form a colony in the PT, CVP or TSA plates used in IFC. The cells may have been killed during the preservation of the slurry. In ELISA, the detection is only based on the reaction of soluble antigens or small cell particles of the target bacterium with the antiserum.

Detectability of Ech and Eca. Isolation on CVP resulted in a detection level lower than 1000 cells of *Erwinia* spp. per ml of cattle slurry. Even when the ratio of *Erwinia* spp. to cattle slurry bacteria was about 1 to 10^5 with pour plating in TSA, the cup-shaped pits could be observed on CVP. These results are about equal with isolation from soil (Cuppels and Kelman, 1974).

For cattle slurry, the average recovery of Ech was 33% lower than that of Eca (Table 3), thus indicating that Ech is more sensitive for toxic effects of the cattle slurry and/or for competition with cattle slurry bacteria than Eca.

With IFC, the detection level was less than 100 cells per ml cattle slurry. Cells need to form a colony in the (selective) agar medium. In the agar medium the colonies stay relatively small and do not overgrow each other. At least 100- to 1000-fold more separate colonies can be formed in the medium than on the medium. Pour plating in PT in combination with immunostaining resulted in a 5 to 10 times higher recovery from cattle slurry in only 0.5 ml PT, compared with traditional surface plating on CVP.

The detectability of ISIF was higher than 10^5 Ech cells per ml, but the presence of falsely positive reacting bacteria may interfere with the reliability of the test.

With ELISA 10^8 or more cfu per ml can be detected in cattle slurry. The detection level of *Erwinia* spp. in cattle slurry is higher than for *Erwinia* spp. in a suspension of the pure culture. This may be caused by proteolytic activity of enzymes from the cattle slurry (Hohmann et al., 1983) on the coating antibodies. Pretreatment of the cattle slurry by filtration and centrifugation to eliminate the large particles in the cattle slurry may increase diffusion and detectability of Ech and Eca in cattle slurry.

Based on the advantages and disadvantages of the various test procedures, IFC is now used to investigate the population dynamics and survival of Ech and Eca inside potato tubers submersed in cattle slurry and of Ech and Eca in the cattle slurry after release from the infected tuber.

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Samenvatting

Vergelijking van immunofluorescentie-koloniekleuring, selectieve isolatie op pectine medium, ELISA en immunofluorescentie-kleuring van bacteriecellen voor het aantonen van Erwinia carotovora subsp. atroseptica en E. chrysanthemi in runderdrijfmest

Verschillende isolatie- en serologische methoden werden vergeleken om de doelbacteriën *Erwinia carotovora* subsp. *atroseptica* (Eca) en *E. chrysanthemi* (Ech) aan te tonen in runderdrijfmest met een natuurlijke bacterieflora van ca 10^8 kolonievormende eenheden(cfu) per ml. De mestmonsters konden gedurende de onderzoekperiode tenminste 8 maanden bij -80 °C worden bewaard zonder dat er een afname van het aantal levende mestbacteriën werd geconstateerd, terwijl bij bewaring bij -20 °C wel een afname werd gevonden. De ontdooide mestmonsters werden geïnoculeerd met de doelbacterie in concentraties tussen 10^2 en 10^8 per ml. De laagste concentratie van de doelbacterie, 10^2 cfu per ml, kon alleen worden aangetoond met de immunofluorescentie-kleuring van bacteriekolonies (IFC) in een selectief medium. Met deze techniek was het percentage herisolatie vanuit drijfmest geïnoculeerd met 10^2 Ech cfu per ml respectievelijk 64% in PT-medium (bevat polygalacturonzuur) en 19% in kristalviolet pectine medium (CVP). Voor Eca bedroegen deze percentages respectievelijk 82% en 32%. In de niet-geïnoculeerde mestmonsters werden geen IFC-positieve kolonies gevonden. Via isolatie op CVP konden 10^3 of meer cfu van Eca en Ech worden aangetoond. Ruwe filtratie van de mestmonsters was nodig voor het aantonen van Eca- en Ech-cellen met immunoadsorptie immunofluorescentie microscopie. De detectiedrempel lag voor deze techniek op 10^5 bacteriecellen per ml mestmonster. In niet-geïnoculeerde mest werden incidenteel IF-positieve bacteriën gevonden. Het aantonen van Ech en Eca met ELISA was slechts mogelijk in mest geïnoculeerd met 10^8 of meer cellen van de doelbacterie per ml.

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