Occurrence and Detection of *Globodera rostochiensis* on Vancouver Island, British Columbia: An Update

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ABSTRACT

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The potato cyst nematode (PCN), *Globodera rostochiensis*, has been present in Central Saanich on Vancouver Island for at least 45 years. Eradication/control efforts have been ongoing, with regulations enacted in the early 1980s restricting the planting of host crops and movement of soil. Surveys monitoring for cyst populations have been minimal since the regulations have been in place with only one limited study in the early 1990s. In this report, a survey of eight fields was undertaken, chosen as the most likely sites that may still harbor viable PCN cysts. Conventional sampling/detection methods were considered inadequate for the detection of very low cyst populations, and an innovative bioassay was developed to improve detection while minimizing costs and labor. Viable cysts were recovered from two fields, both with past quarantine infractions. Fields with no known infractions were found free of viable cysts. Lack of viable cysts found in fields with no infractions suggests that the quarantine restrictions in place since the early 1980s have been effective in reducing or eliminating PCN from these fields. Further systematic and comprehensive retesting of all fields within the quarantine zone is now required, which could lead to the reduction or lifting of some quarantine restrictions.

Potato cyst nematodes (PCN), which include both *Globodera rostochiensis* and *G. pallida*, are highly specialized and successful plant parasites with a long association and co-evolution with their hosts. Their reproductive potential is high, but it is their ability to survive for extended periods under adverse conditions that makes them difficult to eradicate once established. It is the second-stage juvenile (J2) that hatches and exits from the cyst (10), stimulated by host plant root diffusates. Hatching can reach 60 to 80%, but also occurs spontaneously in the absence of a host at an average rate of 30% annually (13). In cold soils, this can be reduced further to 18% (19). J2s invade the host plant and induce cell enlargements which develop into a syncytium, where the nematode attaches and feeds. Females become enlarged as the

eggs develop, and eventually die, while the cuticle tans and forms a protective cyst approximately 0.5 mm in diameter containing 200 to 500 eggs. The cyst eventually detaches and can survive in the soil for long periods of time. There are reports of PCN cysts surviving in field soil for over 20 years. However, soil known to contain 30-year-old cysts has been found to contain only nonviable cysts (18). In Western Australia, in areas infested with PCN between 1986 and 1989 and placed under a strict quarantine and eradication program, viable cysts could not be detected almost 20 years later (3).

G. rostochiensis was detected in western Canada in 1965 within a commercial potato field in Central Saanich on Vancouver Island, approximately 40 km off the mainland of British Columbia, Canada (14). Prior to potato production, the field had been used for several decades for flower bulbs, and it is suspected that the nematode arrived at Saanich on infested flower bulbs imported from Europe. PCN was also found in eastern Canada, in Newfoundland around the same time (12), and more recently in Quebec (17).

Extensive annual field surveys until the early 1980s identified approximately 60 ha of farm land infested with *G. rostochiensis*

(classified as "A"), and another 120 ha associated with the infested lands in Central Saanich (classified as "B"). Eradication/control efforts until 1980 met with limited success, resulting in further restriction on the movement of plants, soil, and farm equipment (9). In 1982, a ban on the commercial production of *Solanum* spp. was enacted in an effort to contain and eradicate the pest (4,15). Of particular concern was the possibility that the nematode could spread to the larger potato growing fields on the British Columbia mainland. The quarantine zone is approximately 3,900 ha and includes residential, rural, and agricultural lands (Fig. 1). With the implementation of the quarantine zone, ongoing survey work was reduced to a minimum and eventually stopped, although enforcement of quarantine restrictions continued. In 1990, a survey was initiated on a limited number of the infested fields. At that time, only one field was found to contain low numbers of viable cysts (D. Kirkham, *personal communication*).

In 2006, we initiated a survey of several well defined sites that had either tested positive for PCN in the past, or were likely to still be infested with PCN. The goal was to actively search for pockets of viable cysts that may still be present in the soil following at least 20 years of quarantine restrictions, and to provide scientific support for the possible relaxation of quarantine restrictions in the area. A secondary objective was the development of a more efficacious and simple method for detection of low PCN populations.

In total, eight fields were selected for testing (Table 1). Historical data, including maps, test and sampling records from the 1960s to the 1980s describing field locations of sites found to be infested with PCN, along with incomplete data on the history of plantings and fumigation, were used to initially identify fields that could still be harboring viable cysts. Following visual inspection, several sites were found unsuitable for sampling because they had been converted to other purposes (kiwi farming, nursery, housing, and meadow) and were impractical for soil sampling.

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Preference was given to fields where viable cysts had been found, for which records indicated no subsequent fumigation had been undertaken or fumigation was incomplete.

The majority of the Central Saanich fields that had tested positive for PCN had no recorded incidences of quarantine infractions over the last 20 years, and had tested negative for viable PCN cysts after the last recorded planting of potatoes. The probability of finding viable cysts at these sites was considered very low, and these fields were not sampled in this study. MAR260 was chosen because, even though it was fumigated in 1984, it was last tested in 1982 with many viable cysts found after the last potato planting in 1981. Viable cysts were also found after the last round of fumigation and potato production in fields MAR209, MAR149, MAR148, MAR71B, and MAR67A. MAR209 most recently had positive test results in 1990 (D. Kirkham, *personal communication*). For the three remaining fields, quarantine irregularities were noted that may have resulted in an increase in the presence of viable PCN cysts. MAR149A, located adjacent to MAR149, was the site of a farm house and associated family yard and garden during the 1960s to 1980s, and was the original site of discovery in 1964. MAR149A was never commercially

farmed, nor tested for PCN, and at some point during the 1990s, it was incorporated into farmland after the building and yard were removed. During the late 1990s, MAR149A was also the site of a small "protest" planting of potatoes. The plants were subsequently discovered by federal inspectors and destroyed, but only after the plants were mature. MAR70A was a family garden plot associated with land infested with PCN. During initial visual field inspections, volunteer potatoes were discovered growing among other vegetables at this location. The owners kept a personal plot of potato plants at that site for several years until they were discovered and removed by federal inspectors. KEA042 was a commercial field also known to have been infested with PCN. During the initial visual site inspection, volunteer potatoes were noted throughout the field. This site had a history of persistent incidences of volunteer potatoes despite ongoing enforcement efforts.

A full survey of the selected fields was not practical, and a random sampling scheme was also discarded based on the low viability count expected. A highly targeted scheme was chosen in order to increase the probability of detection. Distribution of PCN is characteristically patchy (1,5,7); therefore every effort was made to locate previously identified areas

within fields that had tested positive based on historical data. By limiting the area to be sampled, a very high sampling frequency was utilized to increase the probability of detecting viable cysts. Been and Schomaker (1) estimated that using a sampling grid of 5×5 m and a sample size of 6.9 kg/0.33 ha, there was a 90% probability of detecting an infestation focal point density of 50 cysts/kg, or a 100% probability of detecting a focal point density of 150 cysts/kg in potato fields. Since Central Saanich has been free of potato production for at least 20 years, the typical infestation foci were postulated to no longer be present. It would therefore be expected that any foci still present would be diffused due to cultivation, and viable cyst concentrations would be reduced due to natural attrition over time. Therefore, even when targeting known foci, a more intense sampling protocol coupled with a sensitive bioassay would increase the chance of detecting viable cysts.

Plots (10×12.5 m) were strategically positioned over the sampling sites, and a 2.5×20 cm coring probe inserted partway into the soil was used to collect soil samples of *ca.* 50 cm³ each. Within each plot, a core sample was taken on a grid spacing of 0.5×0.5 m, for a total of 500 cores per plot. The 500 cores were pooled (*ca.* 25,000 cm³ of total soil corresponded to a

Fig. 1. Geospatial position of the potato cyst nematode (PCN) quarantine zones in Central Saanich, Vancouver Island, British Columbia. All land east of West Saanich Road is designated class C unless otherwise indicated. Class A and B areas are denoted by shading. In earlier surveys, PCN cysts were found within class A lands. Land designated as class B is associated with class A, but previous testing has not been able to identify PCN.

sampling rate of *ca.* 2,000 kg/ha) and contained in a single 28-liter pot. This sampling frequency was approximately 100-fold greater than that used by Been and Schomaker (1), and more intense than most international PCN sampling strategies (6).

We estimated that the sampling method used had a 95% probability of detecting one viable cyst per 5 liters of soil based on the following calculations. After 25 years of cultivating the upper 25 cm of soil, the distribution of cysts within the foci would be tending toward random (Total volume of soil in the plot = 31.25 m³ with *N* = 500 samples of 0.05 liter taken from each plot). If we assume one viable cyst for each 5 liters of soil and that the bioassay will detect viable cysts every time, then the probability of finding one viable cyst in each 0.05-liter sample is *p* = 0.01; and the probability of not finding a viable cyst is *q* = 1 – *p* = 0.99. Therefore, the mean number of samples with a viable cyst is: *M* = (*N*)(*p*) = (500)(0.01) = 5; and the standard deviation is *SD* = [(*N*)(*p*)(*q*)]^{1/2} = [(500)(0.01)(0.99)]^{1/2} = 2.22. *Z* score = [(*k* – *M*) + 0.5]/*SD* where *k* = 1, the minimum detection level, = [(1 – 5) + 0.5]/2.22 = –1.57, or an estimated 95% probability of detecting one viable cyst/5 liters of soil.

In total, 72 plots were sampled from the eight field locations with the removal of *ca.* 1,800 kg of soil. When KEA042 was sampled, the grower had removed all visible volunteer potato plants the previous day. These plants were found in small piles along the edges of the field. Samples of these piles were also collected.

Direct identification of viable cysts (6,11,16) by crushing, in combination with staining or non-stain evaluative techniques, is unfortunately problematic, and viable eggs/J2s can be missed, or alternatively, live eggs/J2s may be observed but are not capable of reproduction. Cyst extraction from soils is also not completely efficient, especially with soils containing high amounts of organic material. To address these concerns, a bioassay was designed to multiply viable eggs prior to detection. Assuming an optimal healthy cyst with 500 viable eggs, after 20 years and 25%

spontaneous annual hatching, less than two viable eggs would remain. In optimal conditions, two cycles of multiplication should have been sufficient to increase the number of cysts to detectable levels. However, three rounds were chosen to ensure some margin of error. There were two main choices for processing the soil in combination with a bioassay. First, the soil may be processed through a Fenwick can, organic phase collected, contained within a nylon satchel, and placed in a pot of clean soil with a susceptible potato plant. On completion of the bioassay, the satchels are removed and the soil from the pot is again processed using a Fenwick can procedure (8). However, this process is labor intensive with an inherent risk of losing viable cysts during the first extraction procedure. Second, the collected, unprocessed soil may be placed directly within the bioassay containing a susceptible potato plant and grown for several cycles. This would still require processing of the soil in the pot and a distinction made between new, old, viable, and nonviable cysts. To minimize the amount of soil for processing, a modification was made during the last cycle. Instead of planting the potato directly into the pot, the plant was contained in a nylon bag filled with sterilized sand within the pot of soil. The rationale was that cyst nematodes in the soil would be stimulated to hatch and pass through the nylon mesh to the potato roots. Previous results (unpublished) demonstrated that mesh sizes of 75 to 100 µm were effective at containing potato roots, with only minimal root growth of a few millimeters extending through the mesh. The strength of the growing root mass, however, could readily break the stitching of a single mesh bag but was well contained when a double mesh bag was used.

The 73 pots of soil used within the study were placed in a greenhouse with enhanced bio-containment features. Viable cysts were stimulated to hatch and replicate by growing a susceptible potato (cv. Desiree) in each pot for approximately 120 days under the following conditions: 16 to 19 h daylight, day temperature of 18 to 24°C, and night temperature of 12°C.

Plants were from 'British Columbia Elite III certified seed potatoes', and before use were washed and sterilized in 5% sodium hypochlorite. Plants were then removed and the soil air-dried for 2 to 4 weeks before the tubers were removed and the soil mixed. Pots were loosely covered with plastic bags for a dormancy period of 120 days at 6 to 8°C in order for the cyst to undergo diapause (10). The process was repeated two more times. During the third round of multiplication, a double nylon mesh bag (100 µm) containing *ca.* 800 g of sterilized sand and a sterile young potato plant (cv. Desiree) grown from tissue culture was inserted into the soil of each pot. Since roots could grow through the nylon bag several millimeters, it was expected that there might be a higher concentration of cysts in the soil adjacent to the bag than in a control sample taken at the edge of the pot after the final round of amplification. There was also concern that concentrations adjacent to the bag might be higher than inside the bag if root growth was already fully advanced before nematodes began migrating toward the roots. Therefore, at the end of the growing period, the sand, 2.2 kg of soil immediately adjacent to the mesh bag, and a third soil sample of the same size from the edge of the pot were taken and screened for spherical cysts using the modified Fenwick can procedure (8).

Brown-colored spherical cysts with a protruding neck and no protruding terminal cone were observed under low power using a Zeiss Discovery V8 Stereo microscope, and identified as *Globodera* species (10). Cysts were hydrated in water for 3 to 4 days, the cysts crushed using a coverslip, and the J2s assessed under the microscope for viability. Viable nematodes had intact bodies and were mobile. In comparison, dead nematodes were often broken or kinked with disrupted organs. J2s were stained with Meldola Blue to help differentiate live and dead nematodes (11).

Cysts were only found in the mesh bags from five pots from two fields, KEA042 and MAR149A (Table 2). Cysts were not found in samples from two field plots within KEA042; however they were identi-

Table 1. Historical data of fields from Central Saanich, Vancouver Island, British Columbia, surveyed for potato cyst nematode in this study

Field	Year initially tested	Year tested positive	Most recent positive	Number viable cysts	Last year tested	Last year fumigation conducted	Last potato planting
MAR209	1965	1965	1990	36 ^a	1990	1985	1981
MAR149	1965	1973	1986	2	1987	1984	1981
MAR149A ^b	N/D	N/D	N/D	N/D	N/D	N/D	1990s
MAR148	1965	1965	1978	Few ^c	1981	1978	1981
MAR71B	1965	1978	1984	3	1984	1983	1981
MAR70A	1965	1978	1984	1	1984	1981	1980
MAR260	1965	1977	1982	Many ^c	1982	1984	1981
MAR67A	1965	1978	1985	2	1987	1982	1981
KEH042	1966	1982	1982	1	1982	1983	1982

^a After three rounds of biological multiplication in 1992.

^b Anecdotal evidence; N/D = not determined.

^c Direct data entry notation from 1978 and 1982 records. Actual numbers of viable cysts unknown.

fied in the sample containing the discarded volunteer potato plants found surrounding this field. Cysts were found in the mesh bags from all four plots from field MAR149A. From the soil samples, cysts were found in 21 of the 73 pots. In 19 of these pots, the majority of cysts were either broken or puckered and were all classified as nonviable. This was expected, and it confirmed that sampling had targeted areas previously infested with PCN. Viable cysts were found in the soil of four pots. One was from KEA042, the same pot that was found to contain viable cysts in the mesh bag, and the other three were from MAR149A, pots that were also found to have viable cysts in the mesh bags (Table 2).

In pots with viable cysts, more viable cysts were found within the mesh bags than in the adjacent soil. Cyst numbers ranged from 1 to 4,000, and from 2 to 564 for the mesh bag and the soil, respectively. However, the amount of soil sampled adjacent to the mesh bags was almost 2.5 times greater than the amount of sand contained within the mesh bag. In the pot with the lowest number of viable cysts, only one cyst was found within the bag and none were found in the adjacent soil sample.

Comparing the two soil samples from pots with viable cysts, equal numbers or fewer cysts were found in the outer edge of the pot than adjacent to the mesh bag. In no instance were viable cysts detected in the soil but not in the mesh bag. Little difference in cyst numbers was observed at the edge of the pot compared to adjacent to the bag until total cyst numbers increased substantially. This would support the notion of nematodes attaching and developing on roots that pass through the mesh, but would only be of concern at greater population densities and would therefore not affect the results of the bioassay.

To differentiate *G. rostochiensis* from other species, cysts from both suspect fields were analyzed by polymerase chain reaction (PCR) using the PITSr3/ITS5 primer pair which amplifies a 435-bp fragment from *G. rostochiensis* (2,20).

Nucleic acid was extracted from 1 to 5 cysts by grinding in liquid nitrogen. PCR was performed using a Roche LightCycler 1.0 under the following conditions: 50 mM Tris, pH 8.3, 200 ng/μl BSA, 4.5 mM MgCl₂, 0.25 mM each dNTP, 0.5 μM each primer, SYBRgreen (1:50,000 dilution), and 1.0 units of *Taq* polymerase (Roche Diagnostics, Laval, Canada). Cycling conditions were 40 cycles of 95°C for 5 s, 60°C for 5 s, and 72°C for 16 s, preceded by an initial denaturation step of 95°C for 10 min, and followed by a standard melt program step. Amplified products of the correct size were observed from both the MAR149A and KEA042 samples, in addition to the positive control *G. rostochiensis* samples, but not from *G. pallida* (data not shown). Following real-time PCR analysis, fragments were purified using a MinElute PCR column (QIAGEN, Valencia, CA) and cloned into the pCR4-TOPO vector (Invitrogen, Burlington, Canada) as per the manufacturer's instructions. Purified plasmid DNA containing inserts were directly sequenced (University of Victoria, Centre for Biomedical Research). Sequences were assembled using SeqAlign (DNASTar, LaserGene, Madison, WI) and analyzed using BLAST and CLUSTAL W. Sequences obtained from MAR149A and KEA042 cysts were identical to each other (GenBank accession numbers: HM584980 and HM584981) and to several NCBI *G. rostochiensis* sequences, including GQ294512 and FJ212163. Highest identity to *G. pallida* sequences was 95.8% (GU084816) and to *G. tabacum* was 97.4% (GQ294525).

The bioassay was specifically designed for the detection of residual populations of viable cysts in infested soils, and where eradication efforts had been undertaken. The more comprehensive the historical sampling/detection data, the more powerful the assay since only sites within a field known to have contained cysts were targeted and sampled at a very high frequency. The methods could easily be incorporated into standard testing procedures within Canada and the United States for the deregulation of PCN-infested fields

once conventional techniques have failed to detect viable cysts with considerable reductions in costs and labor while maintaining equivalent or higher sensitivity. The process still involves the collection and removal of soil to a contained facility where cysts are multiplied on a suitable host and captured in a sand matrix for easier processing and identification. Ideally, the process would be simplified further by eliminating the soil collection process altogether and inserting the mesh bag with the soil and potato plant directly into the infested fields. Nematodes in the near vicinity would be attracted to the plant and be captured in the sand. However, there is some risk of cysts developing on the outside of the bag on rootlets that manage to pass a short distance beyond the mesh. From the limited data presented herein, this is mainly an issue with high cyst populations. Moreover, these conditions would be unlikely to occur under field conditions where this assay would be implemented.

Of the fields in Central Saanich known to have been infested with PCN, eight were chosen for this survey because they were considered to be of high risk for containing viable cysts. Two tested positive, both with known quarantine infractions. The remaining fields were negative. These fields had no record of infractions with the exception of MAR70A, but still contained viable cysts when last tested. It would appear that 20 plus years in the absence of a suitable host has been sufficient to substantially reduce or eliminate the risk of PCN provided the regulations were upheld. A full survey of Central Saanich is now underway with the possibility that, on completion of the survey, quarantine restrictions can be reduced or in part eliminated.

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Table 2. Number and viability of potato cyst nematode cysts from surveys conducted in the Central Saanich region of Vancouver Island, British Columbia

Field	Pot/plot numbers	Cyst count within mesh bag (ca. 900 g sample)	Cyst count adjacent to mesh bag (ca. 2,200 g sample)	Cyst count at edge of pot (ca. 2,200 g sample)	Cyst viability
MAR209	56 to 72	0	4 (58) ^a , 1 (59), 7 (60), 5 (66), 1 (70)	15 (58), 37 (59), 11 (60)	Nonviable
MAR149	42 to 45	4000 (42), 38 (43), 4 (44), 1 (45)	564 (42), 11 (43), 9 (44), 0 (45)	318 (42), 19 (43), 0 (44), 0 (45)	Viable
MAR149A	46 to 53	0	2 (47), 1 (49), 2 (50), 5 (51), 3 (52)	0 (47), 4 (49), 6 (50), 17 (51), 10 (52)	Nonviable
MAR148	36 to 41	0	3 (36), 6 (37), 1 (38), 9 (39)	N/D ^b	Nonviable
MAR71B	14 to 28	0	1 (15), 2 (17)	3 (15), 0 (17)	Nonviable
MAR70A	54 to 55	0	0	N/D	N/D
MAR260	29 to 35	0	0	N/D	
MAR67A	3 to 13	0	1 (3)	1 (3)	Nonviable
KEA042	1, 2, A ^c	5 (A)	8 (A)	9 (A)	Viable

^a Numbers in brackets are pot/plot numbers.

^b N/D = not determined.

^c Pot A derived from rogued and discarded potato plants found along the edge of the field.

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