PROJECT REPORT No. 340

CEREAL SEED HEALTH AND SEED TREATMENT STRATEGIES: EXPLOITING NEW SEED TESTING TECHNOLOGY TO OPTIMISE SEED HEALTH DECISIONS FOR WHEAT

Technical Paper No. 2

A Real-time PCR Assay for Quantification of *Tilletia tritici* contamination of Wheat Seed

M. McNeil[a], A.M.I. Roberts[b], V. Mulholland[a] and V. Cockerell[a]

[a]*Scottish Agricultural Science Agency, East Craigs, Edinburgh, EH12 8NJ*

[b]*Biomathematics and Statistics Scotland (BioSS), James Clerk Maxwell Building, The King's Buildings, Edinburgh EH9 3JZ*

Submitted to Plant Pathology: 18 December 2003

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INTRODUCTION

One of the most important seed borne pathogens of wheat is 'Stinking' or 'Common' bunt, *Tilletia tritici* ((Berk.) Win 1775 (D.C.) Tul.1847) (syn. *T. caries*). Ears of infected wheat develop 'bunt balls' which replace the seed. These bunt balls, containing millions of spores, are prone to breakage during harvesting and will contaminate the seed lot. Bunt can be easily controlled, since spores contaminating the surface of the grain are relatively easy to disinfect with chemical seed treatments (Paveley *et al.*, 1996). However, in northern and western European countries, expanding environmental awareness and an increase in organic farming over the last 15 years has demanded a reduction in chemical seed treatment. This is causing serious problems for seed producers, who now need alternative methods to control plant diseases, including bunt (Josefsen and Christiansen, 2002; Kristensen and Borgen, 2000).

In the UK, a review of cereal seed health and seed treatment suggested that a move towards 'treatment according to need' would improve both seed health and seed production efficiency (Paveley *et al.*, 1996). However, the current testing methodology for seed-borne pathogens of wheat cannot provide the rapid testing or throughput of samples required for such a strategy.

At present, quantification of *T. tritici* spore contamination involves a seed wash and filtration, followed by microscopic examination for spores trapped on the filter, as described by Cockerell and Rennie (1996). Although this assay can produce results within twenty-four hours, the test is labour-intensive, has a low-throughput, and the morphological identification of spores relies on the skill of experienced analysts. With more than 1000 seed lots being processed per week during the autumn, it is recognised that the development of a high-throughput quantifiable test is required for the adoption of a treatment according to need strategy.

There is increasing interest in the application of polymerase chain reaction (PCR) technology for the identification of plant pathogenic fungi, as this technology reduces or eliminates the need for lengthy culturing and difficult morphological identification procedures (McDonald *et al.*, 2000). PCR has also been used to improve the sensitivity of fungal pathogen detection on seed (Ophel-Keller *et al.*, 1999), and many assays have been developed to quantify the amount of fungal pathogen present (Böhm *et al.*, 1999, Bates *et al.*, 2001, Edwards *et al.*, 2001, Mulholland and McEwan, 2000, and Smith *et al.*, 1996). Of these, only Bates *et al.* (2001) and Ophel-Keller *et al.* (1999) have developed or are developing a method of real-time PCR that will quantify the amount of fungal pathogen on seeds.

A competitive PCR method was developed to quantify *T. tritici* (Mulholland and McEwan, 2000), but the use of this multi-step process involving gel electrophoresis to measure pathogen DNA, makes it an inefficient choice for high throughput testing. In this study, we report the development of a real-time PCR to determine *T. tritici* contamination in samples of wheat seed. Real-time PCR uses a fluorescence detection system to measure a cycle threshold (Ct) value that can be transformed into a quantity of DNA after comparison to a DNA calibration curve. This data can be compared to the *T. tritici* contamination given by microscopic analysis, to enable results to be expressed as an equivalent number of spores/seed. To improve the accuracy of real-time PCR, TaqMan® chemistry (as described by Böhm *et al.* 1999) has been incorporated into the assay. This chemistry utilises a specific probe labelled with a fluorescent dye to detect the formation of PCR product during the PCR amplification.

MATERIALS AND METHODS

Seed samples

Samples of wheat, submitted to the Official Seed Testing Station (OSTS) Scotland for routine *T. tritici* analysis, were tested in duplicate using the real-time PCR assay, described in this paper, and the microscopic assay described by Cockerell and Rennie (1996).

Seed was sampled and prepared using the hand halving method in accordance with the International Rules for Seed Testing, (Anonymous, 2003); three, 300 seed replicates were prepared for each assay.

Chlamydospores

Chlamydospores were obtained by harvesting ears from a bunt-infected crop sown at East Craigs, Edinburgh, from certified wheat seed artificially contaminated with *Tilletia tritici* chlamydospores. Bunt balls (sori) were manually removed from ears and crushed through a series of sieves, to remove debris and allow chlamydospores to be collected. These chlamydospores were then used for *T. tritici* DNA extraction procedures.

Chlamydospores from seven different species of *Tilletia* known to occur in the UK and Europe, including *T. tritici* were sourced according to table 2.

DNA Extraction from seed

Each seed replicate was placed into a 50ml conical centrifuge tube, and 20ml of 1× 10mM Tris, 1mM EDTA (TE) buffer pH8 (100× TE, Sigma, diluted in HPLC grade water, Sigma) was added. The tubes were then placed onto a flask shaker and agitated for 5 minutes. The resultant solution was decanted into a 50ml conical centrifuge tube. A further 20ml of 1× TE buffer was added to the seeds and the tube contents were vortexed for 10 seconds. The resultant solution was decanted into the tube with the first 20ml, combining the two washes. The total wash extract was centrifuged at 3076g for 15 minutes to pellet any spores and the supernatant decanted into another 50ml centrifuge tube. The resultant pellet was dispersed in 1ml of the decanted solution and transferred into a 2ml microtube. The microtube was centrifuged at 11500g for 15 minutes and then all the supernatant was removed by pipetting. The resultant pellet was dispersed in 200µl of the liquid. To release DNA from any *Tilletia* spores present, the suspension was rapidly frozen at -70^{o}C, then heated to 90^{o}C. The DNA extract obtained was centrifuged at 11500g for 5 minutes in a microcentrifuge. 150µl of the liquid was then transferred by pipette into a 200µl microtube, and centrifuged at 11500g for 5 minutes to give a suitable template for the PCR assay. For the first 41 samples tested, each of the 3 replicates were tested individually. Thereafter, for all samples, 50µl from each replicate was combined prior to being used as a template.

DNA Extraction from *T. tritici* Chlamydospores and quantification

DNA from *T. tritici* spores was extracted using the method described by Gang and Weber (1995). The resultant stock solution of purified *T. tritici* DNA was quantified spectrophotometrically using a GeneQuant RNA/DNA calculator (Amersham Biosciences).

Samples of chlamydospores from other species of *Tilletia* and *Ustilago* (Table 2) were suspended in 1× TE buffer, and a simple freeze/thaw extraction method was used to release the DNA. This was necessary, as there was an insufficient quantity of spores to allow a pure DNA extraction. Extracted samples were centrifuged at 13000rpm for 5 minutes before being used as a template for PCR.

Fungal Isolates

DNA extracts from 29 common seed-borne fungi were used to check primer/probe specificity. Fungal isolates from pathogens likely to infect wheat seed or plants were chosen, along with a range of other common agricultural fungal pathogens and saprophytes (Table 2). Pure colonies from both the fungal reference collection at the OSTS and pure colonies isolated from samples submitted for testing were used. These were plated aseptically onto potato dextrose agar (PDA) plates, containing streptomycin, which had been covered with a circle of cellulose film. Colonies were grown at 18^{o}C for 5 days, before being extracted using a modified version of the DNA extraction protocol in Genescan Europes' (Germany) 'GMOScreen Advanced Screening System Basic' protocol (Standard method). The modification included the addition of an RNase, followed by a further phenol/chloroform extraction. The DNA extracts were then quantified spectrophotometrically using a GeneQuant RNA/DNA calculator (Amersham Biosciences).

Real-time PCR Method

The real-time PCR assay incorporated TaqMan® chemistry, using a sequence-specific probe hybridising between the forward and reverse primers, as described by Böhm *et al.* (1999). The samples were amplified and analysed using an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Warrington).

For each sample a specific Ct value was calculated. The Ct value is defined as the cycle number at which a statistically significant increase in the ΔRn (the difference between the reacted sample and unreacted sample) is first detected. This is also the cycle at which the detection of target crosses an arbitrary threshold. The threshold is defined as the average standard deviation of ΔRn for early cycles. It is placed where the detection of an increase in signal associated with an exponential growth of PCR product has occurred. The Ct values thus obtained are then used to calculate the initial amount of DNA present.

A method of absolute quantification was used, comparing the amount of DNA in unknown samples to that of known DNA Standards. The slope and intercept of the regression of the Ct values for standards, on their known DNA quantities (after taking logarithm base 10) were used to calculate the amount of DNA for each seed sample. These DNA amounts were converted into an equivalent number of spores per seed using calibration curves.

Primer and Probe design

Real-time PCR primers were designed to amplify a short segment or amplicon, 69 base pairs long, of the target nucleotide sequence from the *T. tritici* 25S ribosomal RNA subunit gene (Accession number L20285). A probe labelled with a fluorescent dye was also designed to anneal to a sequence between the primer pair. These primers and probe were designed using PRIMER EXPRESS® SOFTWARE (Applied Biosystems, Warrington). The National Centre for Biotechnology Information's (NCBI) BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) was used to search the GenBank nucleic acid database for sequences from closely related species. The sequence chosen for primer and probe design was aligned with these sequences to judge specificity of amplification. No significant matches were revealed with other species in the database. Primers and probe used were, forward primer Tc-F, reverse primer Tc-R and probe Tc-Pr (Table 1). The TaqMan® probe was labelled at its 5' end with the fluorescent reporter dye TET™, whereas its 3' end was modified with the universal quencher dye TAMRA™. Applied Biosystems, Warrington, provided synthesis of oligonucleotides and probe labelling.

Fungal and chlamydospore DNA extracts were tested, prior to testing with TaqMan® primers and probe, for the presence of fungal DNA using non-specific general fungal oligonucleotide primers FUN28-A and FUN28-B (Table 1) together with SYBR® Green PCR Master Mix (Applied Biosystems, Warrington).

Table 1 Primer and Probe Sequences

Primer	Sequence (5'→ 3')	Source
Tc-F	TTGGGATTGGCGTATTTGC	This work
Tc-R	ATGCCACATTTCTCCTACTATTATCCA	This work
Tc-Pr	TET- TTGTGCAGGCCAGCATCGGTTTT - TAMRA	This work
FUN28-A	CCAGAGACCGATAGCGCACA	Mulholland, McEwan (2000)
FUN28-B	TGCGAGCCTCCACCAGAGTT	Mulholland, McEwan (2000)

PCR Conditions

All real-time PCR assays were performed in an ABI Prism 7700 sequence detection system (Applied Biosystems, Warrington).

T. tritici real-time PCR reactions were performed in duplicate in a final volume of 25 µl. Real-time quantitative reactions were performed in MicroAmp® optical 96-well plates and closed by MicroAmp®optical caps (Applied Biosystems, Warrington). Each 25µl reaction contained, 1µl template (various amounts of DNA), 8µl of HPLC grade water (Sigma), 12.5µl of 2× TaqMan® Universal Master Mix (Applied Biosystems, Warrington), 7.5pmol of each primer and 2.5pmol of probe. The samples were amplified for 40 cycles of 95°C for 15 seconds, 60°C for 1 minute, preceded by a 95°C incubation for 10 minutes. Two wells of negative controls, containing no template, were included with each sample assay.

The general fungal primers FUN28-A and FUN28-B were used to amplify fungal DNA together with 2× SYBR® Green PCR Master Mix. Extracts were amplified using the same cycle program for TaqMan® primers described above.

Check of DNA Standards

The stock *T. tritici* DNA solution was serially diluted using 1× TE buffer, to give standards ranging from 1000ng to 0.05ng DNA (final amount in PCR reaction). The quantity of DNA encompassed by these standards covered the range of contamination levels likely to be observed within samples submitted to the OSTS for testing.

Creation of Calibration Curves

Calibration curves were created using samples with a known contamination level, as assessed by the microscopic test method described by Cockerell and Rennie (1996). For this purpose, a calibration set was constructed based on 26 seed samples with contamination levels ranging from 0 to about 1000 spores per seed. A replicate from each sample was assayed in each of three separate runs (each run containing 26 samples), and within the assay each PCR reaction was duplicated. The logarithms (base 10) of DNA quantities, for the samples, were calculated using the standard curves and averaged over the three runs. Calibration curves were estimated by the linear regression of the logarithm of the spores/seed plus 1 on the mean $\log_{10}$ DNA quantities over all the samples. Predictions and prediction intervals for the contamination levels of further samples could then be calculated (Draper and Smith, 1981)

based on Ct values. To evaluate the accuracy of these predictions, 226 samples submitted to the OSTS, were tested by the microscopic and real-time PCR methods.

RESULTS

Check of Primers and Probe specificity

Using the general fungal primers, DNA was found to be present in all fungal and chlamydospore DNA extracts.

The TaqMan® primers and probe were found to be genus-specific for *Tilletia*, but not species-specific for *T. tritici* (Table 2). No amplification was observed for any other species of fungal pathogen tested with this primer/probe combination.

Calibration of real-time PCR for absolute quantification

Ct values for the standards (obtained from DNA extracts of chlamydospores) in the calibration runs are shown in figure 1. Some systematic run-to-run variation was evident, and a regression of Ct on the logarithm base ten ($\log_{10}$) DNA quantity, grouped by run, showed evidence for differences in intercepts (p-value=0.001), but not for difference in slopes (p-value=0.80) between runs (Table 3).

Ninety nine percent of the variation is explained by the straight-line relationship, and individual intercept and slope values are within 0.63 and 0.12 respectively of each other.

The infection levels for the set of calibration samples as assessed by the microscopic method are shown in table 4. DNA was extracted from these samples, used as a template for real-time PCR, and Ct values were obtained. Dropout Ct values, where no amplification had occurred, were then eliminated.

Table 2 Results of real-time PCR primer and probe specificity tests against pure fungal DNA extracts.

No	Fungal Isolates	Reference[a]	Source[b]	Origin	Result[c]
1	*Ascochyta sp.*		1	UK	-
2	*Pyrenophora teres*	FRC004	1	Aberdeenshire	-
3	*Pyrenophora avenae*	FRC005	1	Orkney	-
4	*Ascochyta pisi*	FRC010	3	Cambridge	-
5	*Ascochyta fabae*	FRC008/CABI 135517	2	UK	-
6	*Alternaria sp.*		1	UK	-
7	*Phoma betae*	FRC012/CABI 156653	2	UK	-
8	*Fusarium venetatum*	CC392	1	UK	-
9	*Alternaria brassicae*	CC379	1	UK	-
10	*Pyrenophora graminea*	FRC003	1	Mid-Lothian	-
11	*Penicillium sp.* (1)		1	UK	-
12	*Septoria nodorum*	FRC007	1	Perthshire	-
13	*Fusarium sp.*		1	UK	-
14	*Penicillium sp.*(2)		1	UK	-
15	*Fusarium avenaceum*	CC 260/CABI270166	2	UK	-
16	*Microdochium sp.*		1	UK	-
17	*Fusarium graminearum*	CC269/CABI263190	2	UK	-
18	*Phoma pinodella*	FRC009	3	East Anglia	-
19	*Mycosphaerella pinodes*	FRC011	3	Cambridgeshire	-
20	*Botrytis sp.*		1	UK	-
21	*Fusarium culmorum*	FRC002	1	Chichester	-
22	*Cochliobolus sativus*	FRC006	1	Perthshire	-
23	*Colletotrichum*	CC406	3	Lincolnshire	-
24	*Phomopsis sp*		1	UK	-
25	*Epicoccum sp*		1	UK	-
26	*Microdochium nivale*	FRC001	1	Perthshire	-
27	*Ustilago tritici*		7	Cambridgeshire	-
28	*Ustilago nuda*		1	UK	-
29	*Ustilago avenae*		6	Norway	-
30	*Tilletia controversa*		4	Canada	+
31	*Tilletia foetida*		4	Canada	+
32	*Tilletia laevis*		5	Iran	+
33	*Tilletia lolii*		5	Iran	+
34	*Tilletia holci*		5	NZ	+
35	*Tilletia sphaerococcus*		5	Italy	+
36	*Tilletia tritici*		1	Edinburgh	+

[a] FRC, OSTS Fungal Reference Collection; CC, OSTS Culture Collection; CABI, CABI Reference

[b] 1, OSTS, Edinburgh, UK; 2, CABI, Wallingford, Surrey, UK; 3, PGRO (Processors and Growers Research Organisation), Peterborough, UK; 4, J. Sheppard, Central Seed Laboratory, Ottawa, Canada; 5, P. Piepenbring, Botanisches Institut, Univesitat Tubingen, Germany; 6, G. Brodal, Norwegian Seed Testing Station, Ås, Norway; 7, J. Thomas, NIAB, Cambridge, UK.

[c] Presence or absence of amplification product: - absent, + present

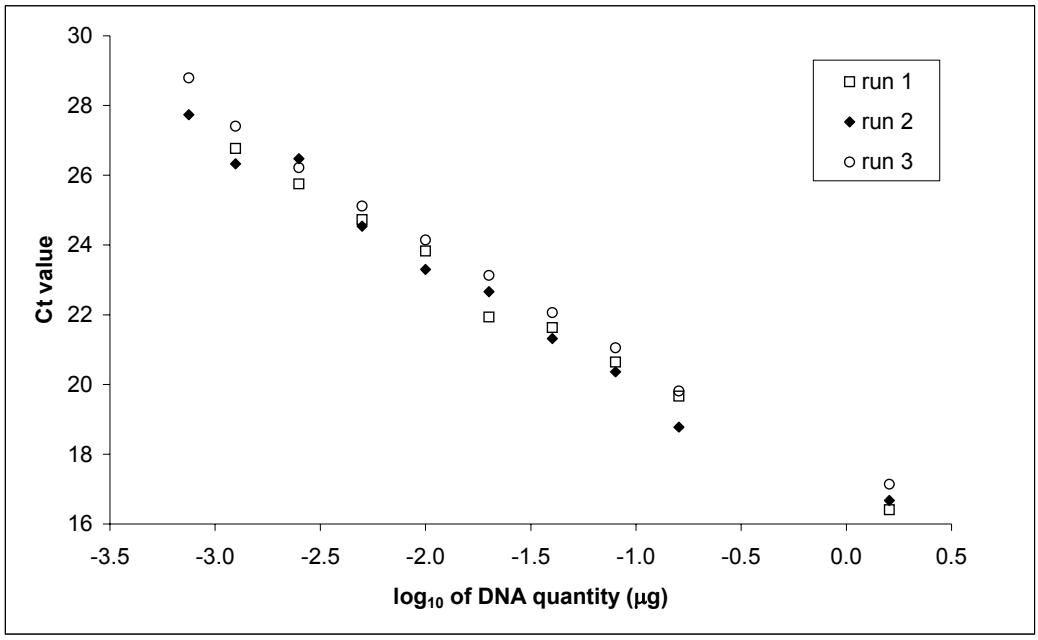

Figure 1 Means of duplicate Ct values plotted against µg DNA for the standards in the calibration runs.

Table 3 Coefficients of determination (R^2), intercepts and slopes for the individual regressions in the three runs

Run	R^2	Intercept	Slope
1	99.3%	16.95	-3.34
2	98.3%	16.72	-3.44
3	99.3%	17.35	-3.46

Ct values for these samples were converted to DNA amounts (µg) for the three runs using the slopes and intercepts in table 3. A plot of the $\log_{10}$ DNA (µg) and $\log_{10}$ spores per seed (after adding 1) is given in figure 2. Regression of $\log_{10}$ contamination levels on $\log_{10}$ DNA (µg), grouped by run, showed no evidence for differences in intercepts (p-value=0.12) or slopes (p-value=0.19) between the runs.

Table 4 Contamination levels for calibration set samples and their Ct values as obtained in the real-time PCR calibration runs.

		Ct values		
Sample Number	Number of Spores/seed	Run 1	Run 2	Run 3
1	0.00	32.70	30.80	31.34
2	0.25	30.32	29.99	30.18
3	0.50	29.95	29.39	28.67
4	1.66	29.87	29.84	27.82
5	3.23	27.60	27.78	26.39
6	4.47	28.14	28.16	27.36
7	5.30	28.11	29.21	28.31
8	6.38	28.63	29.17	26.97
9	8.29	27.70	27.48	26.08
10	10.80	28.00	27.87	27.25
11	19.89	32.97	36.61	35.33
12	25.11	23.88	27.05	24.03
13	28.67	25.91	26.89	24.94
14	37.29	40.00*	40.00*	40.00*
15	45.95	25.13	25.15	24.55
16	50.15	24.81	25.43	24.10
17	58.84	23.02	23.50	22.72
18	58.67	26.32	26.19	40.00*
19	68.66	25.45	25.55	24.24
20	154.90	24.40	23.84	22.52
21	332.40	23.43	24.71	23.01
22	450.24	20.44	24.90	19.80
23	678.47	20.68	20.69	20.40
24	849.27	20.01	21.14	19.35
25	965.61	22.37	22.18	21.45
26	1074.00	20.64	22.04	20.28

For each sample, the $\log_{10}$ of the quantity of DNA (µg) was averaged over the three runs. The calibration model was formed, by regressing the $\log_{10}$ (number of spores/seed +1) on the mean $\log_{10}$ DNA (µg). The sample with zero spores per seed was omitted, as PCR results for this low contamination level did not fit the linear model. The R^2 for this regression was 90.4%, the intercept was 3.95 and the slope was 0.988.

Predictions of infection levels for a range of DNA quantities with 95% prediction intervals are shown in figure 3.

* Dropout Ct values where no amplification occurred

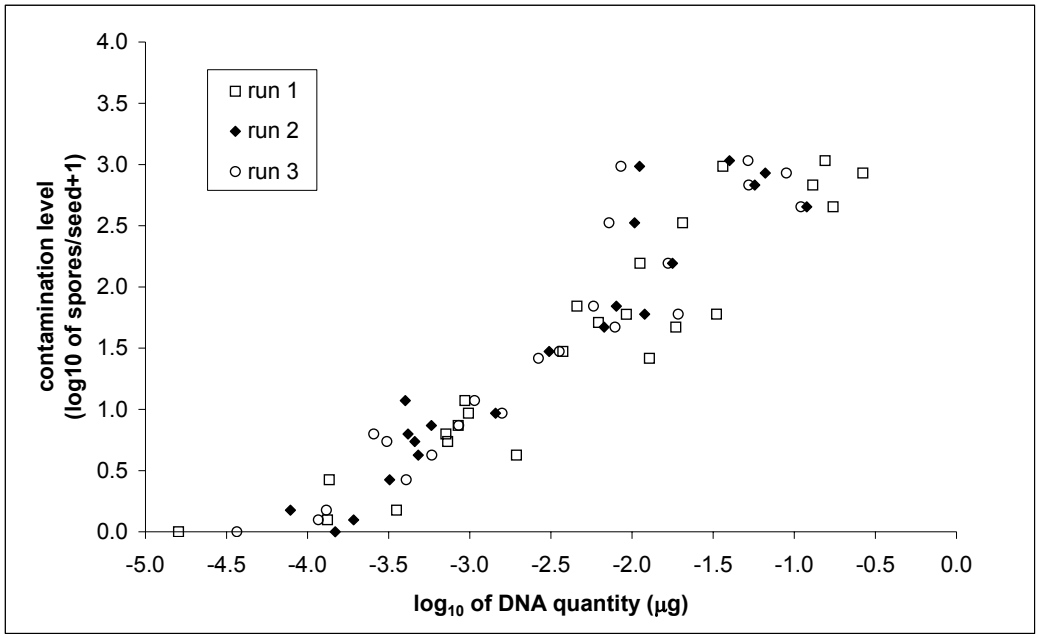

Figure 2 A plot of the contamination level determined microscopically for the calibration set samples against DNA amounts obtained in each of the three runs.

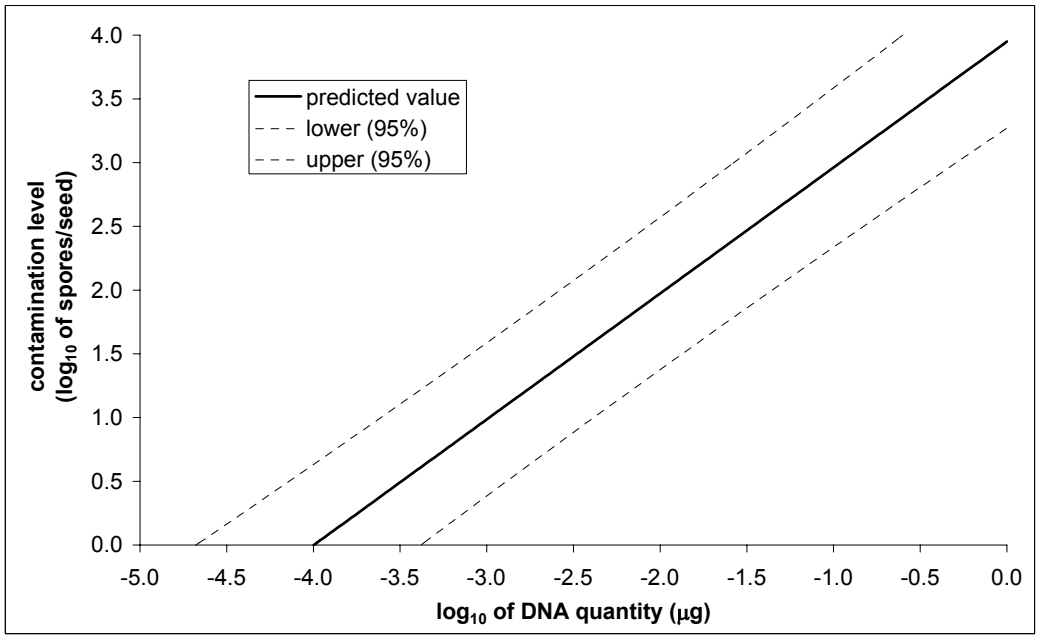

Figure 3 Calibration model showing predictions of infection level based on DNA quantities with 95% prediction intervals.

Prediction of contamination levels for 226 seed samples submitted to the OSTS for testing and comparison with microscopic assay results

Samples were tested over 10 real-time PCR assays and with the conventional microscopic assay. The coefficient of determination R^2, intercept and slopes for the standards in each of the 10 runs are shown in table 5.

Table 5 Coefficient of determination (R^2), intercepts and slopes of the Standards in the test runs

Test run	R^2	Intercept	Slope
1	99.6%	17.98	-3.70
2	99.5%	17.46	-3.96
3	99.2%	17.87	-3.79
4	99.3%	17.65	-4.15
5	99.4%	17.87	-4.05
6	99.5%	17.71	-4.03
7	98.8%	17.24	-4.13
8	98.8%	17.24	-4.13
9	99.7%	17.38	-4.15
10	99.3%	17.68	-4.25

For repeat assays of Standards, an average 99.3% of the variation is explained by the straight line relationship, and intercepts for all runs differed by no more than 0.74, and slopes by a maximum of 0.55.

The contamination levels for the test set samples were predominantly low, with 53% having zero spores per seed and 70% having less than 1 spore per seed (Table 6).

Table 6 Contamination level frequencies in seed samples used to test real-time PCR assay.

Spores per seed (assessed by microscope)	Number of samples
0	119
> 0 & ≤ 0.5	25
> 0.5 & ≤ 1	14
>1 & ≤ 2	15
>2 & ≤ 5	17
>5 & ≤ 10	10
>10	26

Dropout Ct values, where no amplification had occurred were eliminated. The Ct values for the test samples were converted to DNA quantities using the slopes and intercepts in table 5. The contamination levels and DNA quantities, for both calibration and test samples, are shown in figure 4. The test runs produced higher DNA measurements than the calibration runs for samples with similar contamination levels assessed by microscopic assay.

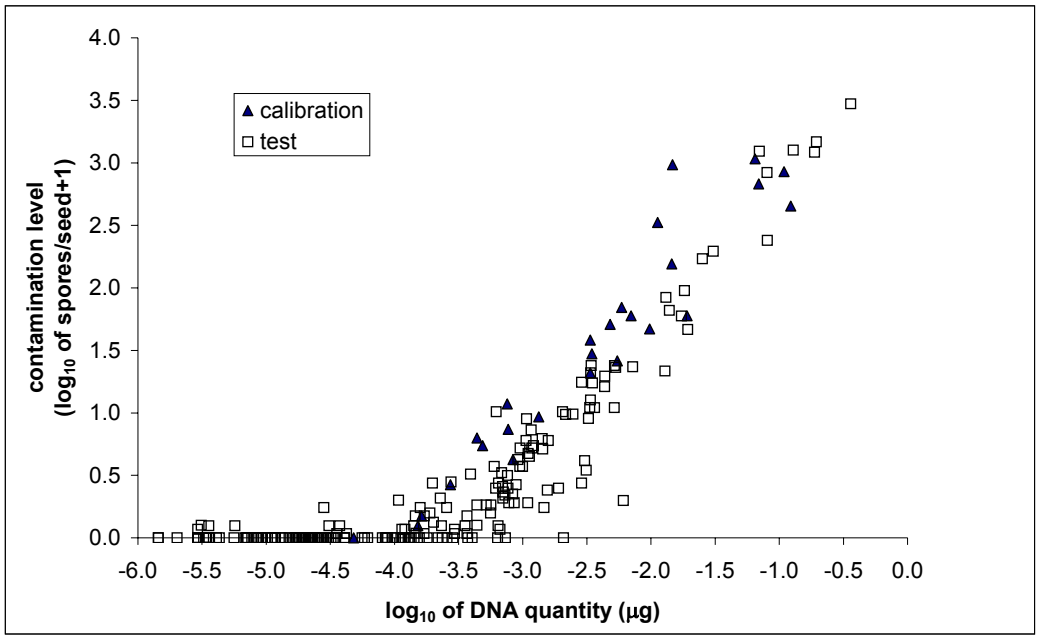

Figure 4 A plot of contamination level against DNA quantity for both calibration and test samples.

Using DNA quantities, predictions of contamination level were made for each test sample based on the calibration model (Figure 3). The calibration model successfully included 91% of samples within the 95% confidence intervals. This is slightly less than expected for a 95% interval (two-sided binomial test p-value=0.005). Of the 21 samples out-with the confidence intervals, 16 represented over-predictions of contamination level. The five under-predictions were all for samples with low levels of contamination (<1 spore per seed) and may reflect the clear signs of non-linearity observed in figure 4 for samples with low contamination levels. In general, there was a tendency to over-predict contamination levels (Figure 5).

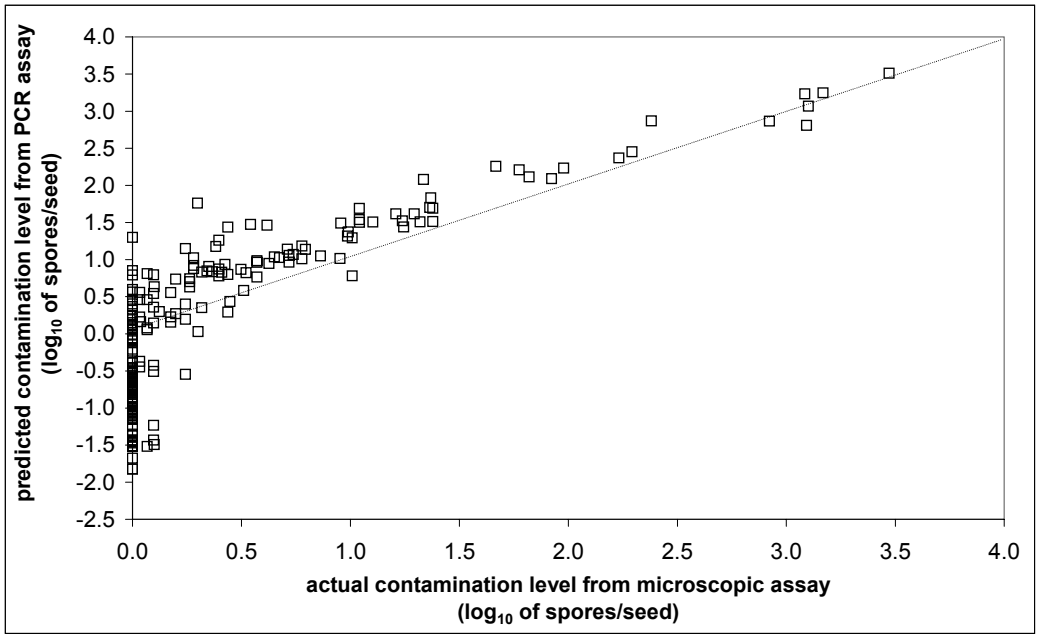

Figure 5 Plot of predicted versus microscopic contamination levels for test samples (Diagonal line shows where predictions equal actual results).

Threshold Levels

Another way of assessing the results is by comparison with a threshold. The quantity of DNA that has an upper (95%) prediction interval of 1 spore/seed was 44 pg. Introducing a threshold level of 1 spore per seed, the prediction intervals were used to select samples that would be likely to have less than 1 spore per seed. Of the 226 samples, 99 had less than 44pg and of these, none had more than 1 spore per seed by the microscopic assay. On the other hand, 57 with less than one spore per seed in the microscopic assay had more than 44pg DNA.

Evaluation of run-to-run variation

It was clear from the comparison of calibration results with test results that there were systematic differences between the calibration and test runs (Figure 4). To examine further the extent of the run-to-run variation, repeat real-time PCR assays were conducted on the three sets of test samples that had the widest range of contamination levels (runs 7, 8 and 9). The results from the original and repeat runs were compared using t-tests on the DNA quantities ($\log_{10}$ transformed) from each batch, paired by sample. There was evidence for systematic differences between the original and repeat runs for all three batches (p-values <0.001 for run 7, <0.001 for 8 and 0.065 for run 9). However, the direction of these differences was not consistent between batches, with run 7 showing an increase in DNA

measurements (mean difference of 0.13 $\log_{10}$ (µg)), and runs 8 and 9 showing a decrease (mean differences of 0.22 and 0.07 $\log_{10}$ (µg) respectively).

DISCUSSION

A real-time PCR assay, using TaqMan® chemistry, has been developed for the quantitative analysis of *T. tritici* DNA. The results of this assay can be expressed in terms of the number of spores present per seed using a calibration model, and can also be used in conjunction with thresholds to inform decisions regarding seed treatment.

In real-time PCR, amplification of the PCR product is followed throughout the reaction, and samples can be differentiated at the exponential point of amplification, where there is greatest separation between different levels of DNA. This test is faster and more accurate than the competitive test developed by Mulholland and McEwan (2000) that relies on end-point analysis. Within the real-time PCR assay, the use of TaqMan® chemistry, which uses a specific probe, increases the specificity of the reaction. It gives greater sensitivity than a real-time assay using a non-specific DNA binding dye, and allows the detection of *T. tritici* contamination levels to less than 1 spore/seed.

The real-time PCR assay uses primers Tc-F and Tc-R and detects all *Tilletia* species that may be found on wheat seed in the UK. The regions of *Tilletia* DNA that have already been sequenced show few dissimilarities between species, and it has not been possible to design primers from these to differentiate between different species. Josefsen and Christiansen (2002) were also unable to design species-specific primers for the common bunt species *T. tritici*, *T. laevis*, *T. controversa* and *T. bromi*, as there was no variation between the sequenced ITS regions in these species.

The possibility of these other *Tilletia* species being detected on wheat seed was examined and a variety of sources were consulted (Crop Protection Compendium – Global Module, 1999 Edition, CABI; Descriptions of Fungi and Bacteria, CABI; Distribution Maps of Plant Diseases, CABI/EPPO) to ascertain the status of the non-target species in the UK. The current risk, of obtaining a false positive result using this primer-pair within an assay, was assessed as negligible since the probability of other *Tilletia* species occurring at any significant level within UK wheat seed samples is remote.

With the acquisition of a greater knowledge of the gene sequences of different *Tilletia* species, species-specific primer/probe combinations may be designed, which would allow differentiation between the *Tilletia* species. In the pursuit of such primers, McDonald *et al.* (2000) used genetic fingerprinting techniques to successfully reveal differences between groups of *Tilletia* species. Smith *et al.* (1996) used oligonucleotide primers, designed after mitochondrial DNA sequence analysis, to distinguish *T. indica* from *T. barclayana*, and in 2000, Frederick *et al.* differentiated *T. indica* from the morphologically similar species *T. walkeri*, using specific primers designed from nucleotide differences within the mitochondrial DNA region.

When using the microscopic assay it is also very difficult to differentiate with absolute certainty between different species of *Tilletia*. Even when scanning electron microscopy is used to examine chlamydospore ornamentation, some genera appear similar (Piepenbring *et al.*, 1998). If specific real-time PCR primers for *T. tritici* were developed, the analytical skills required for pathogen species identification would not be required.

When comparing repeat runs of DNA Standards and sample extracts, there is greater variation between repeat assays on the same seed sample extract than is found in repeat assays of the same purified DNA extract. This may be due to the different methods of DNA extraction. As *T. tritici* spores are present on the outer surface of the grain, a crude but rapid method of DNA extraction has proved sufficiently adequate to allow the detection of spores to a level of <1 spore per seed. The DNA extraction method used for standards however, has several purification steps within the methodology. These extra steps should mean that these purified DNA extracts have less contamination than the crude extracts obtained from seed samples, and this could be the reason for the consistency of repeat tests of standards. Other potential sources of variation within the real-time PCR assay such as; sampling, dilution of the seed extract, and pipetting techniques, were investigated (data not shown), and the introduction of quality assurance procedures to the test aims to minimise these possible sources of variation.

In addition to the variation in extraction methods and the variation within the real-time PCR assay, there is the potential for variation due to the distribution of *T. tritici* contamination within seed lots. Law *et al.* (2003) found Bunt distribution in some seed lots to be heterogeneous and with such lots variability between repeated real-time PCR and/or microscope tests would be expected.

Although the predictions based on this calibration have been successful, the results do indicate some areas where there could be some improvement in the calibration model.

Incorporating the run-to-run variation that existed between the test runs and the current model would give more appropriate prediction intervals. The calibration model did not adequately accommodated the imprecision associated with both PCR measurements of DNA quantity and microscopic evaluations of contamination level; there is likely to be both measurement error and variability due to sampling. Examination of the DNA values for samples with low infection levels indicated that there might also be a sensitivity problem for low DNA quantities. Roberts, Theobald and McNeil (unpublished) have now incorporated these factors into an improved Bayesian model.

Due to the level of variation present within the current model, the test should only be used to decide whether the sample being tested is above or below a given threshold level. Thresholds for bunt are being considered using a conceptual model of plant infection and spore release (Cockerell *et al.*, 2003). The decisions, to treat seed or not for conventional farming practice, are based on an advisory threshold of 1 spore/seed, and may also be appropriate for decisions regarding sowing or rejecting a seed lot for organic farming.

When the predicted real-time PCR results were compared with microscopic assay results only two samples, that were found to be above the threshold, by the microscopic test, were predicted to be below the threshold by the real-time PCR assay. The calibration model has been designed to minimise the chance of a sample remaining untreated with a spore loading of 1 or more spores per seed. Where the predicted level was greater than or equal to 1 spore/seed, 45% had a spore loading of less than 1 spore/seed when assessed using the microscopic assay. However, 91% of samples fell within the 95% confidence interval of the calibration model.

The real-time PCR assay for *T. tritici* has several advantages over the microscopic test method. It is less labour-intensive, the number of samples that can be assayed at one time is increased and this allows an increased throughput of samples. There is no requirement for morphological identification of *T. tritici* spores, which requires lengthy and time-consuming training. This real-time PCR assay can detect levels of bunt contamination down to less than 1 spore/seed, which is sufficiently sensitive to be used in conjunction with current thresholds, allowing a quick decision to be made regarding treatment of the seed lot.

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