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Method Verification of the LOD Associated with PCR Approaches for the Detection of Horse Meat

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Summary

In 2013, the Department for Food and Rural Affairs (Defra) and the Food Standards Agency (FSA) commissioned a UK Survey of beef products as part of a co-ordinated response to the EU horse-meat issue. Samples were taken on a formal basis, allowing UK Public Analysts to choose which methods to apply. A range of analytical methods were available for detection of horse DNA, but the respective Limits of Detection (LOD) were often different, not robustly defined, or expressed using different measurement units. The LOD of methods used in the UK Survey needed to be robustly tested and qualified so that results obtained from the samples could be interpreted with confidence.

The aim of the present study was to evaluate the LOD of three selected methods used by Public Analysts as part of the UK horse-meat Survey in terms of uniform w/w (raw horse-meat in a raw beef (meat) background) sample measurements. The three methods evaluated were a PCR-Capillary Electrophoresis approach (PCR-CE as described in Defra project FA0220, LOD reported as approx 1% w/w); PrimerDesign (LOD of approx. <100 mitochondrial copies); and Neogen BioKits (LOD approx 0.1% w/w).

A range of gravimetrically prepared raw horse-meat in raw beef meat (w/w) materials were produced as part of the current study and authenticated for species identity. These materials were used to challenge the three methods in order to estimate the LOD in terms of w/w (meat to meat) based on internationally accepted guidelines and best measurement practice for LOD and PCR methods. Estimates for the LOD were based upon 60-115 replicates of the 0.1% w/w material, depending upon the method evaluated. More than 250 replicates of the 0.1% w/w material were assessed across the three analytical methods, representing five independent DNA extractions.

Results showed that all three methods were capable of reaching an LOD of less than 0.1% w/w raw horse-meat in a raw beef (meat) background if Quality Procedures and Good Laboratory Practice for molecular biology methods were adhered to. This helped afford good comparability of results for these three methods, and in turn contributed to ensuring that the results from the UK Survey of beef products in 2013 were interpreted with confidence.

Keywords

Horse meat, Limit of Detection (LOD), PCR, real-time PCR, Capillary electrophoresis, Defra project FA0134

Introduction

Food authenticity issues and food fraud are becoming increasingly problematic owing to pressures on food production and the current climate of financial constraint. The findings of horse DNA present in beef burgers sold in a UK supermarket chain in 2013[1] highlighted the need to provide support for rapid and reliable appraisal of the meat supply chain by developing standardised approaches for the detection and quantitation of different meat products.

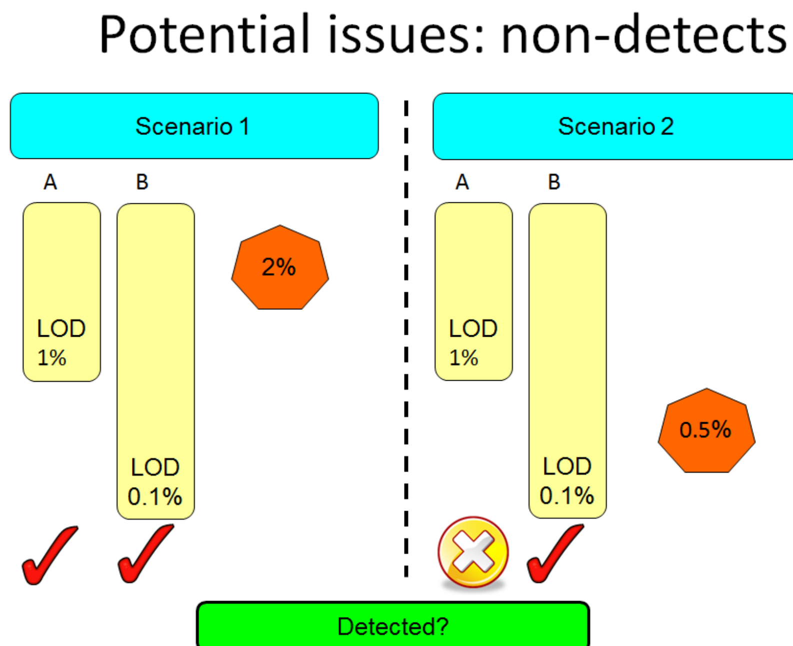
In response to the horse-meat issue, the Department for the Environment, Food and Rural Affairs (Defra) and the Food Standards Agency (FSA) conducted a UK wide Survey for detection of equine DNA in food samples[1]. Because samples were taken under formal sampling conditions, the Public Analyst laboratories taking part in this survey were free to choose whichever method they felt appropriate for detection of horse DNA. There is currently a wide choice of methods (published papers and commercially available kits) that are available for the detection of horse DNA. However, the limit of detection (LOD) of these methods is variable and had not been robustly defined, with results often expressed in different measurement units (e.g. DNA/DNA, DNA copy numbers, pg of DNA, mitochondrial or nuclear DNA, meat in a meat background on a weight for weight basis [w/w]).

The LOD is an important performance characteristic of a method that needs to be evaluated through method validation. This limit needs to be robustly tested and qualified so that results from the UK Survey can be interpreted with confidence. The LOD of a method can be defined as the lower limit of applicability of an analytical approach[2]. If different methods are characterised as having different LOD's, then they will not be performing to the same level. Those methods that have higher (less stringent) LOD's than other methods may have a higher probability of not detecting the target when that target is at a very low concentration. An example of this failure to detect the target is illustrated in Figure 1. If two methods have LODs of 1.0% (w/w) (e.g. raw horse meat in a raw beef background) and 0.1% (w/w) then a sample with 0.5% (w/w) target species content may remain undetected by one method, but will be identified and characterised as detected using the other method (Figure 1).

The two methods used for detection have different LOD's. Method A has a LOD of 1% w/w (e.g. raw horse meat in a raw beef background) and Method B has a LOD of 0.1% w/w. In Scenario 1 a test sample containing 2% w/w raw horse meat in a raw beef background will be detected by both methods. In Scenario 2 the test sample contains 0.5% w/w of the target and hence will only be reliably detected by Method B. This serves to illustrate why it is important to have the same LOD associated with different methods applied as part of an inter-laboratory

analysis, and also highlights the importance of evaluating performance characteristics as part of the validation of a method.

Figure 1 – Diagrammatic Representation of the LOD



Establishment of a LOD would be supportive of provision of a solid framework and infrastructure for implementing a "threshold" for detection of equine DNA, relative to the w/w meat for meat equivalent, which is thought to be due to deliberate adulteration as opposed to adventitious contamination. Such an approach has been discussed and advised as appropriate in the European Commission Recommendation of 27 March 2014 (2014/180/EU)[3] which describes a second coordinated control plan to help establish prevalence of fraudulent practices in the marketing of certain foods.

The aim of the current study was to use a statistically robust experimental design with sufficient levels of replication to fully assess the LOD associated with three methods for detection of horse DNA used as part of the Defra/FSA horse-meat survey in 2013. The methods evaluated were the PCR capillary electrophoresis approach (as described in Defra report FA0220[4] and hereafter referred to as PCR-CE/FA0220), and commercially available kits of Neogen BioKits[5] and PrimerDesign[6] for the detection of horse DNA. Establishment of a robust LOD for these methods would allow the results from the Defra/FSA survey on detection of equine DNA in food samples to be interpreted with confidence. This will also provide support for policy makers to implement an appropriate threshold setting if applicable, above which presence of horse DNA is thought to be due to deliberate adulteration and not simply due to adventitious contamination.

Materials and Methods

Sourcing of Materials

Meat samples were sourced from reputable suppliers inclusive of those that had the appropriate accreditation to international standards and maintained traceability records. Reference materials consisting of raw muscle flesh, trimmed free of surface inter-muscular fat and connective tissue were produced, which were authenticated as to species identity using real-time PCR, ELISA and DNA sequencing.

In 2013 LGC produced reference materials of raw horse muscle flesh and mixtures of raw lean horse in beef, to be used as quality control material for Public Analysts as part of the beef product survey. The material was prepared gravimetrically by weighing the required amounts of authenticated raw horse meat into authenticated raw beef (meat) and placed into individual 50mL screw-cap sample pots. When stored under the recommended conditions (minus 20±5°C), the materials were expected to remain stable for at least six months. Tests were carried out at LGC to check the effect of one additional freeze-thaw cycle and no significant change was observed on analysis. Gravimetric preparations of 100%, 30%, 10%, 5%, 1%, 0.5% and 0.1% (w/w) of raw horse meat in a raw beef (meat) background, were produced.

DNA Extraction and Quantitation

To emulate a range of DNA extraction approaches that could be used as part of the beef product survey, a generic in-house DNA extraction approach was used. One gram of sample was added to 5ml of buffer containing SDS and 0.5mg/ml of Proteinase K. This was vortexed and incubated at 55°C for 1 hour to achieve sample lysis. A proprietary DNA binding buffer using positively charged silica beads was added to the sample and incubated for 10 minutes at room temperature to achieve DNA binding, followed by a series of multiple washing steps at 2500rpm for 2 minutes. DNA was then precipitated in ethanol and left for 30 minutes at 55°C before being eluted in 1ml of an elution buffer. Yield and quality of the extracted DNA (OD 260:280) were checked using a Nanodrop ND-1000 Spectrophotometer. Five independent DNA extractions were represented at the 0.1% (w/w) level.

PCR Methods

Three different methods of PCR analysis which were used as part of the original beef-product survey in 2013 were evaluated. These included an FSA-approved PCR-Capillary Electrophoresis method (PCR-CE/FA0220), a Neogen BioKits qualitative method and a PrimerDesign quantitative method.

The PCR-CE/FA0220 qualitative approach is described in detail in the Defra FA0220 report[4] and other publications[7,8,9] and includes primer sequences for several mammalian and bird species. However, in the present study the LOD was determined using only the assay for horse DNA. Singleplex PCR was carried out with 50ng DNA in a volume of 25µl using a

Multiplex Master Mix (Qiagen) and a PE9700-9 thermal cycler. Thermal cycling conditions were used as recommended by Qiagen, and included 45 cycles of denaturation/annealing/elongation. In terms of the LOD, the original method stated this to be approximately 1% w/w[4].

The Neogen BioKits (HE mastermix pod) method was carried out as specified by the manufacturer[5], using the mastermix provided and AmpliTaq Gold® DNA Polymerase (Applied Biosystems), as specified in the protocol. The master mix contains two assays for duplex amplification of a horse-specific DNA target and an unspecified mammalian housekeeping gene target. 50-100ng DNA was added to a total reaction volume of 25µl, and the recommended cycling conditions were followed using a PE9700-9 thermal cycler. The Neogen BioKits protocol states that the LOD of the method is approximately less than 0.1% w/w[5].

The PrimerDesign real-time PCR approach was conducted using TaqMan Universal PCR Mastermix (Life Technologies) on the ABI Prism 7900HT Real Time PCR system. The primer and probe mix supplied with the PrimerDesign kit was reconstituted in nuclease-free water, prior to the addition of 1µL mix per reaction. Replicate 20µl reactions were prepared, containing 25ng of genomic DNA extracted from 0.1% w/w horse meat in beef. These were run alongside the *Equus caballus* positive control DNA provided with the kit, encompassing the range of 10^2 to 10^6 copies mitochondrial DNA per reaction as an approximate guide for DNA amount. Thermal cycling conditions were: 50°C for 2 minutes, 95°C for 10 minutes, followed by 50 cycles of: 95°C for 15 seconds, 60°C for 15 seconds. The LOD of the PrimerDesign method was stated as approximately 100 copies of mitochondrial DNA[6].

Table 1 provides a summary of the distribution of each of these methods across the Public Analyst (PA) laboratories involved in the detection of horse meat.

Table 1 - Summary of the PCR Methods used by Public Analyst Laboratories for the Detection of Horse Meat in the 2013 Survey of Beef Products

Number of Laboratories	Method	LOD
3	PrimerDesign "Quantitative real time PCR"[6]	Approximately 100 copies mitochondrial genome
2	Neogen BioKits "Qualitative PCR"[5]	Approximately <0.1% w/w
2*	PCR-CE/FA0220 (qualitative)[4]	Approximately 1% w/w
1*	FSA: Chisholm *et al.*, 2005 "Real time qualitative PCR"[10]	25pg of mitochondrial DNA
1	Real-time semi-quantitative PCR (in-house)	<0.01% w/w

The above table illustrates the range of methods, LOD's and LOD expression units quoted as part of the method used for the UK 2013 beef product survey. Eight Public Analyst laboratories took part in this survey. One laboratory used two methods and these are denoted by the asterisk (*).

Analysis of PCR Amplicons

PCR amplicons were assessed by capillary electrophoresis using a DNA1000 reagent kit on an Agilent Bioanalyzer 2100 for both the PCR-CE/FA0220 and Neogen BioKits methods. Targets from horse DNA were considered as detected by observation of an amplicon at the appropriate theoretical size (82bp and 331bp respectively for the two methods). Cross-contamination of reactions was tested for by running a PCR no-template control (water substituted for DNA template) on each DNA chip.

The Neogen BioKit protocol recommends analysis of PCR amplicons via electrophoresis on a 3% agarose gel. However, the laboratory using the Neogen BioKits method as part of the UK survey of beef products confirmed that they had used the Agilent Bioanalyzer, so it was deemed appropriate to mirror this method in the present study so that the results were more representative.

Analysis of PCR products for the PrimerDesign method was conducted using real-time PCR software (SDS) as described by the kit manufacturer[6]. DNA from horse was detected when a sample well was assigned a Ct value within range of a dilution series produced by the positive control supplied with the kit. PCR no-template controls were also included in the analysis to test for any cross-contamination.

Specificity of Methods

For the PCR-CE/FA0220 and PrimerDesign methods, species cross-reactivity was examined to assess the specificity of the method for the horse DNA target. The horse-specific assay was tested against various animal DNA templates including goat, deer, turkey, chicken, donkey, lamb and duck which were obtained as pre-extracted genomic DNA from a commercial supplier (AMS Biotechnology Limited). For the Neogen BioKits method, specificity data was taken directly from the manufacturer's protocol and was not tested as part of the current study.

Measurement Units Used

The LOD was expressed in terms of w/w raw horse meat in a raw beef (meat) background.

Establishment of an LOD

The LOD of a method can be defined as the lower limit of applicability of an analytical approach. The LOD may be specified as the lowest concentration of target analyte that can still be detected on 95% of occasions[2]. The LOD can be evaluated using a range of

approaches. For example, the LOD can be characterised by experimentally determining the proportion of replicate analyses where the target is detected in a serial dilution series of the target. Alternatively, a Probability of Detection approach (POD), based on modelling the probability of detecting the DNA target in a more limited range of concentrations and as first described in Burns & Valdivia (2008)[2] and elsewhere in the published literature[11] can also be utilised.

Results

DNA Extraction and Quantitation

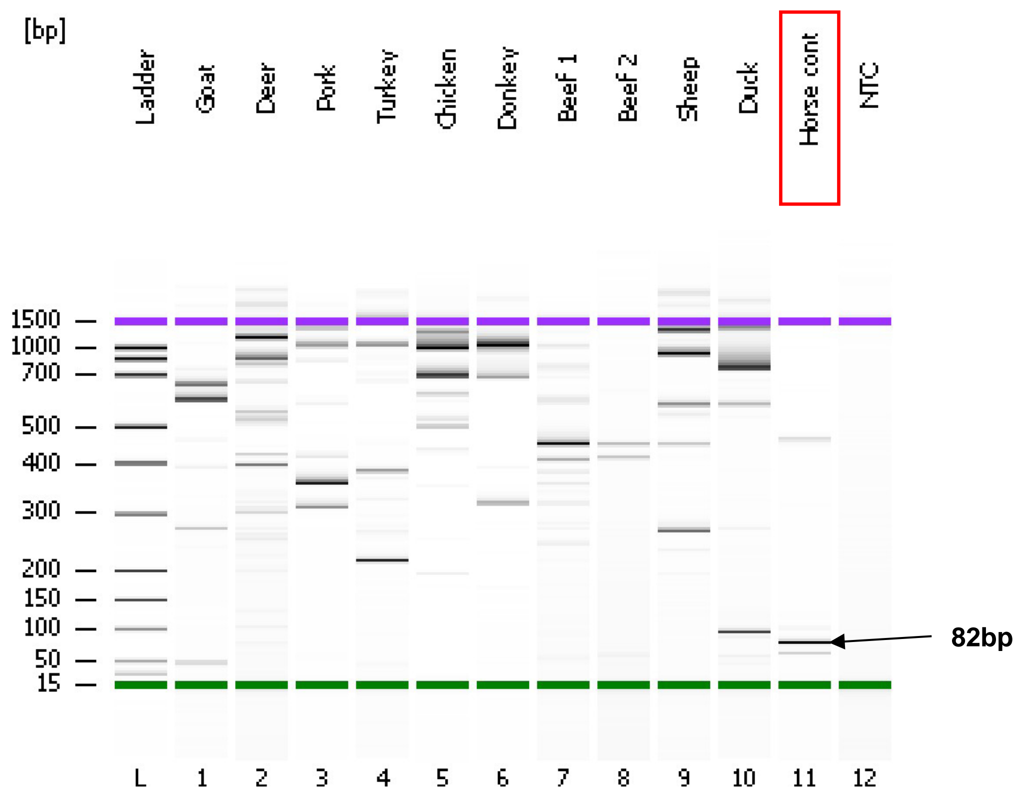
1g of sample was used per extraction replicate, which was eluted in 1ml total volume of elution buffer. The average yield across all horse meat-containing samples (including 100% horse) was 164.28ng/µl with a standard deviation of 24.66ng/µl. Optical densities (OD 260:280) ranged from 1.69 to 2.44 (data not shown). The mean DNA yield for the 0.1% w/w horse in beef samples was 167.27ng/µl with a standard deviation of 27.88ng/µl. The mean optical density at OD 260:280 was 2.17. For the 0.1% (w/w) gravimetric standard, five independent DNA extractions were taken.

Specificity of Methods

Specificity was assessed for the PCR-CE/FA0220 method (Figure 2). Animal species including deer, pork, chicken, donkey and sheep were included in the analysis, where each species template was tested against the horse-specific assay. Despite a high degree of non-specific amplification being observed for all species with multiple bands greater than 82bp, the amplicon that was indicative of horse DNA at 82bp was only present in the horse control following electrophoresis. This indicated that the PCR-CE/FA0220 method was specific for horse in this format.

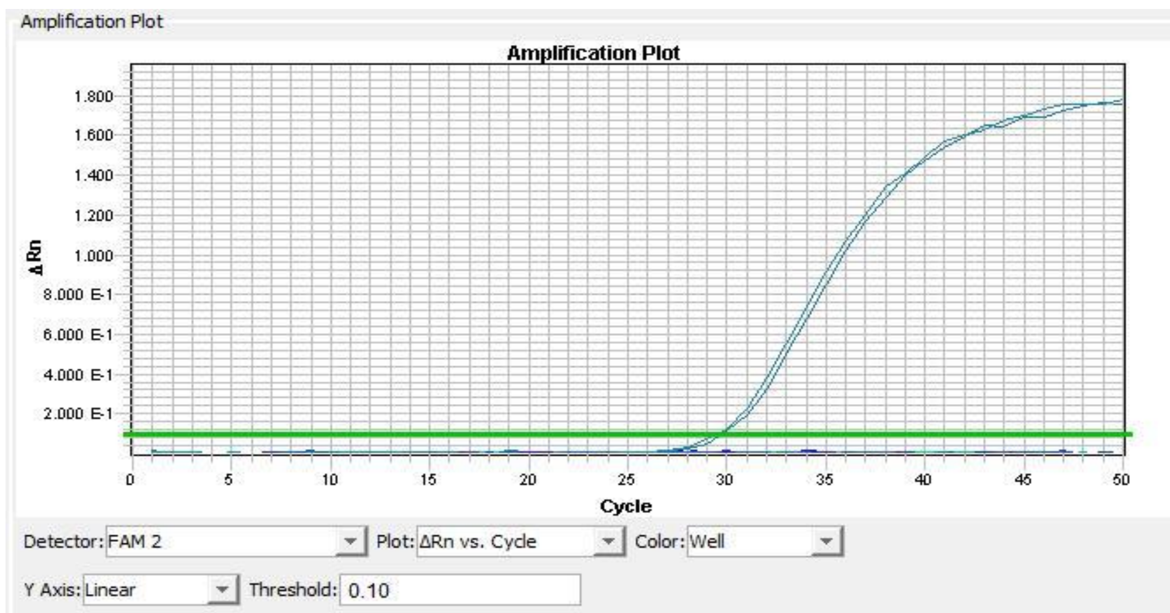
Cross reactivity tests were also carried out for the PrimerDesign real-time PCR method across the same set of species DNA templates (Figure 3). None of the alternative templates were amplified and none were assigned Ct values, whilst the horse genomic DNA positive control was assigned a mean Ct value of 29.74 and a standard deviation of 0.20. This indicated that the PrimerDesign method was specific for the horse DNA target.

Figure 2 - Gel-like Image following Cross-Reactivity Tests using the PCR-CE/FA0220 Method



The above shows example results of the cross reactivity tests from the PCR-CE/FA0220 approach on the Agilent Bioanalyzer. Lanes 1 to 11 represent PCR products from the different DNA species templates, and lane 12 represents a PCR no-template control. A base pair ladder is included in the lane marked “L”. Multiple bands are present in lanes 1 to 11 but note that the band at 82bp is present exclusively in the Horse control (lane 11). This is indicative of the horse DNA target being detected by the assay.

Figure 3 – Linear Amplification SDS Plots following Cross-Reactivity Tests with the PrimerDesign Method



The above shows a real-time PCR amplification plot associated with the specificity tests for the PrimerDesign assay. Cycle number is shown on the x-axis and change in fluorescent response is shown on the y-axis. Note that only the horse positive control was amplified by the PrimerDesign *Equus caballus* PCR kit. No other templates were amplified, including the PCR no-template controls and reactions contacting DNA from the other species standards.

Assessment of the LOD Associated with the PCR Methods

Preliminary studies were carried out for the PCR-CE/FA0220 method using DNA ad-mixtures of horse DNA in a beef DNA background to assess the applicability of the assays in detecting horse DNA in a beef DNA background. Dilutions were made of the following ratios of horse DNA to beef DNA: 50%, 10%, 1%, and 0.1%. A positive control of 100% horse genomic DNA was also included in the study. Results showed that all DNA:DNA ratios were detectable by the assay, with a band appearing at 82bp that was indicative of horse DNA being present.

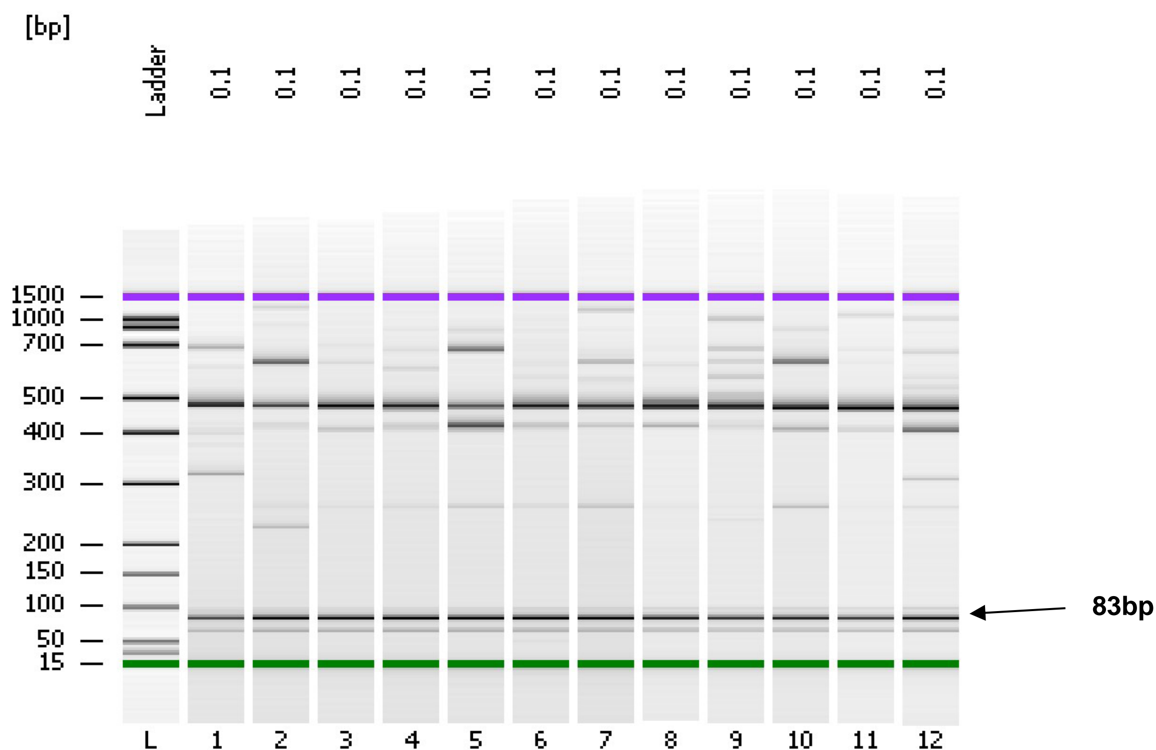
Following this, DNA was extracted from the range of gravimetrically prepared materials (100%, 30%, 5%, 1%, 0.5% and 0.1% w/w of raw horse-meat in a raw beef background) and applied to the three different PCR-based methods to determine the LOD. For each of the methods used in the present study at least 60 PCR replicates (representing at least five independent DNA extractions) of 0.1% w/w horse meat in beef were tested in order to determine the LOD. All three methods were successful in detecting the horse DNA target at the different dilution levels, including those at 0.1% w/w. This was repeatable across all independent DNA extractions. Because all replicate reactions at all of the percentage w/w levels, inclusive of the 0.1% (w/w) level, were detected by all three methods, it was not necessary to apply a Probability of Detection approach to the data. Table 2 provides a summary of the number of replicates used at the 0.1 w/w% level, the lowest in the test series, for each of the three methods included in the present study.

Table 2 - Summary Results for the 0.1% (w/w) Replicates for Detection of Horse DNA in the three PCR Methods (PCR-CE/FA0220, Neogen BioKits and PrimerDesign)

0.1% Horse in beef (w/w) method	Number of PCR replicates at the 0.1% level	Total number of PCRs where horse DNA was detected	Theoretical Amplicon size	Observed Amplicon size
PCR-CE/FA0220	115	115 (100%)	82bp	83bp
Neogen BioKits	61	61 (100%)	331bp	343bp
PrimerDesign	91	91 (100%)	N/A	N/A

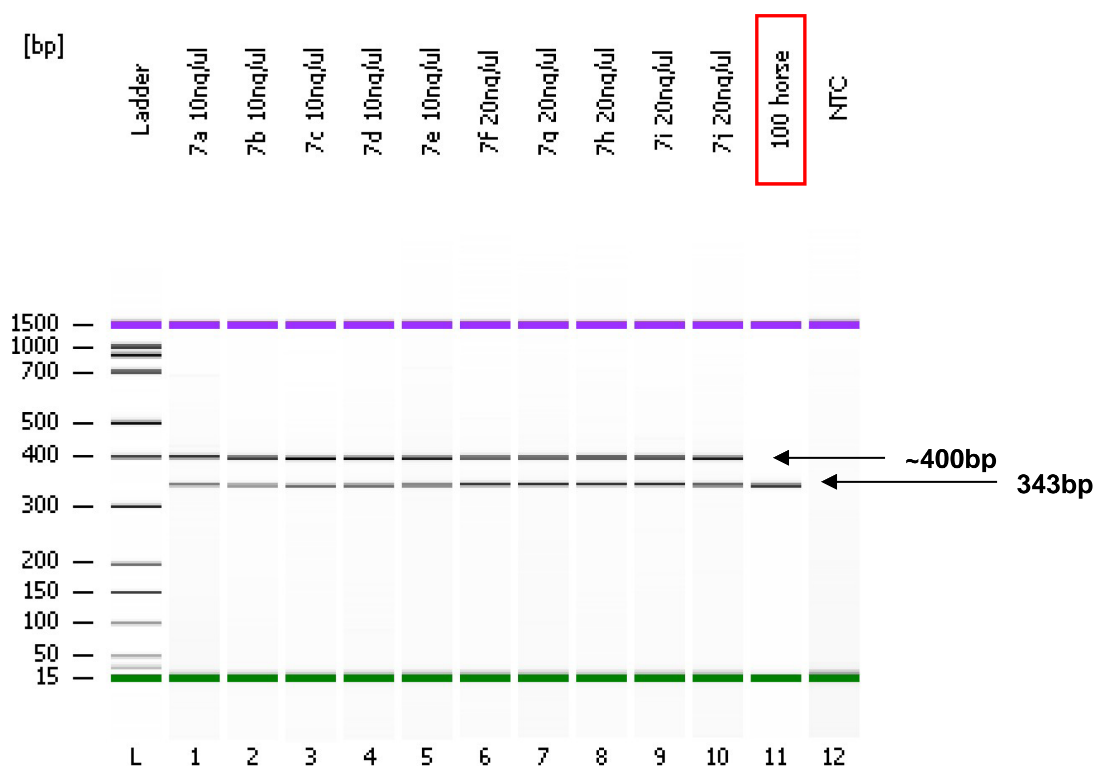
The above table shows the number of PCR replicates tested representing the 0.1% (w/w) raw horse meat in a raw beef background gravimetric standard, for each of the three methods investigated as part of this project. For all three methods, one hundred percent of the PCR replicates at the 0.1% (w/w) level had a detectable response for the horse target.

Figure 4 - An Example Gel-like Image of the Results for the PCR-CE/FA0220 Approach



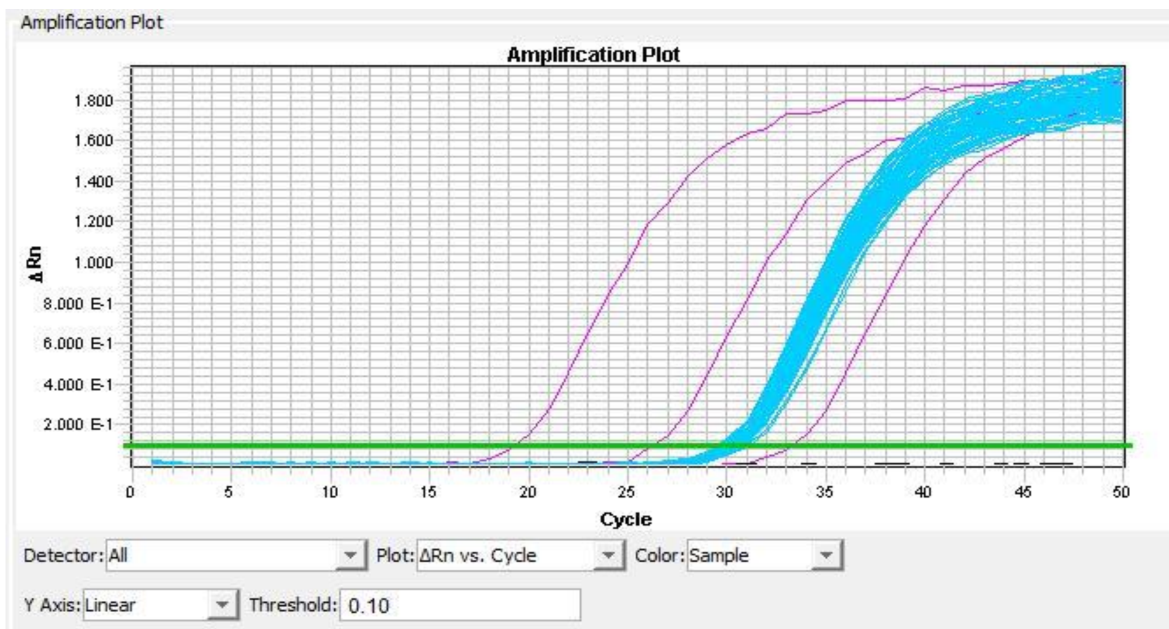
Lanes 1 to 12 represent individual PCR replicates of the 0.1% w/w horse in beef standard. Note the band at 83bp indicative of horse DNA target being detected by this assay. Additional bands are also present at 66bp and 470bp, which may be due to the extended cycle length, or due to another target being detected by the horse assay. A separate chip was run containing only positive and negative controls from the same 96-well PCR plate to ensure that the assay was fit-for-purpose (not shown).

Figure 5 - An Example Gel-like Image of the Results for the Neogen BioKits Approach



Lanes 1 to 10 represent individual PCR replicates of the 0.1% w/w horse in beef standard. Note the horse specific band at 343bp. In addition, a mammalian housekeeping gene is included and observed at around 400bp. This housekeeping gene is often not seen in the 100% horse positive control (lane 11), which may be due to the high concentration of horse DNA out-competing the mammalian housekeeping gene target during primer hybridisation. Lane 12 contains a PCR no-template control (water substituted for DNA template).

Figure 6 - Linear Amplification SDS Plots for 25ng 0.1% w/w Horse in Beef Using the PrimerDesign Method



A real-time PCR amplification plot associated with testing the PrimerDesign assay against the 91 PCR replicates derived from the gravimetric 0.1% (w/w) raw horse meat in a raw beef background standard. Cycle number is shown on the x-axis and change in fluorescent response is shown on the y-axis. The light blue lines indicate amplification from all 91 PCR replicates of the 0.1% (w/w) standard. The three purple lines are the *Equus caballus* positive control standards supplied as part of the kit, at amounts of 10^6, 10^4 and 10^2 horse mitochondrial copies per reaction going from left to right.

Discussion

The in-house DNA extraction method based on cell lysis with SDS, binding of DNA to positively charged beads, and subsequent washing, ethanol precipitation and elution stages produced extracted DNA of reasonable yield and quality. This in-house method was chosen so as to emulate DNA representative of different yield and quality metrics, as the DNA extraction approach used by the laboratories in the original UK survey of beef-products was not prescribed.

The aim of the current study was to determine the Limit of Detection (LOD) of three methods used by UK Official Control Laboratories as part of the 2013 UK Survey of beef-products for the presence of horse-meat, in order to establish if these methods had comparable performance in terms of lower limits of applicability. Traditional chemical methods for estimating a LOD are based on the determination of blank "negative control" values. These take into account the mean measurement response from the negative controls, as well as a derivation of their standard deviation. However, data sets generated from molecular biology methods that use the Polymerase Chain Reaction (PCR) approach often do not fulfil the assumption of normality, exhibit truncated data, and are often heteroscedastic (the variability of the data set is related to its mean value)[2]. For these reasons, the negative control cannot be routinely used to determine the LOD for PCR methods, and instead a more practical solution had to be sought. The LOD can also be defined as the lowest concentration of a target analyte that can still be detected with a 95% probability[2]. In order to facilitate the establishment of the LOD, a range of gravimetrically weighted raw horse meat in raw beef (meat) samples were prepared at the 100%, 5%, 1%, 0.5% and 0.1% w/w levels. These w/w standards were applied to the three different PCR-based methods to determine the methods' LOD. All three methods were successful in amplifying the appropriate target sequence in all DNA samples. A total of 267 PCR replicates were tested at the level of 0.1% w/w horse in beef level across five different sample extracts.

The first method assessed by this study was the FSA validated PCR-CE/FA0220 method[4], which is a qualitative method designed for the detection of DNA from a range of animal species commonly consumed by humans. These species included horse, donkey, duck, deer, pheasant, turkey, chicken, pig, cow and sheep. For the purposes of LOD estimation in the present study, only the assay for horse was used. In total, 115 replicates from five different DNA extracts were assessed by this method. PCR products were run on the Agilent Bioanalyzer 2100, and a band around the expected 82bp was observed. This confirmed that horse DNA was detected in all 115 replicates for this method. PCR no-template controls for the target were always included in order to test for possible cross-contamination from horse DNA; in all cases these came up as negative. In addition to the expected 82bp amplicon, other bands at 66bp and 470bp were commonly observed following electrophoresis with this assay (Figure 4). However, these products were often less abundant than the target amplicon, and may have been a result of the increased cycle length. The amplicon at 470bp may be a result of non-specific hybridisation of the primers to other parts of the genome. For the purposes of detection of a product that has theoretically and experimentally been shown to represent the present of horse DNA in a sample, these additional bands are not significant.

The second approach assessed was the Neogen BioKits method[5]. In comparison to the PCR-CE/FA0220 method, a range of assays were available for the detection of various different animal species including horse, pork, beef, sheep, chicken, turkey, goat and rabbit. However, only the horse-specific assay was of interest for the purpose of defining the LOD for this method. A total of 61 replicates of 0.1% w/w horse in beef were tested using this assay, which all showed detection of the horse DNA target with an amplicon of approximately 331bp.

The third method assessed was the PrimerDesign quantitative PCR approach[6]. 91 replicates of the sample 0.1% w/w horse in beef sample were assayed alongside varying dilutions of the positive control DNA provided with the kit. The primer design method was successful in characterising the LOD in terms of less than 0.1% (w/w) horse in beef for all replicates at this level.

The results of the present study indicate a high level of repeatability across all three methods for the detection of horse DNA target in the 0.1% w/w horse in beef standard. Overall, the results of the present study provide strong evidence that the LOD for each of the methods can be interpreted as less than 0.1% w/w horse meat in beef, indicating that each are equally as effective in the detection of horse DNA target in meat samples at this level.

This measurement unit of w/w raw horse meat in a raw beef (meat) background was chosen as it appears to be the measurement unit most often quoted and has practical implications in terms of visualising and providing “meat in meat” materials. Whilst scientifically and metrologically the expression units of DNA copy numbers is more traceable, the use of this measurement expression has little bearing on practical measurements in meat samples, and to attempt to establish such a link between w/w and DNA/DNA copies was beyond the remit of this current project. The expression of a LOD in terms of w/w tissue also facilitates the public’s interest in understandable research results, conforms with the European Union’s view of defining threshold limits for prohibited species in terms of percentages of the meat content (w/w), and may facilitate a need to control product labels that express meat content on a w/w basis[3].

Conclusion

The results have shown that the three approaches of the PCR-CE/FA0220 method[4], the Neogen BioKits method[5], and the PrimerDesign method[6] all have the potential and capability of reaching a limit of detection (LOD) of less than 0.1% w/w raw horse meat in a raw beef (meat) background. Providing that laboratories apply Quality Procedures and Good Laboratory Practice for DNA extraction and application of molecular biology methods, it is reasonable to expect that the LOD of <0.1% (w/w) should also be readily achievable.

All three methods assessed as part of the study (PCR-CE/FA0220, Neogen BioKits, PrimerDesign) have an LOD of <0.1% (w/w) of raw horse meat in a raw beef (meat) background. There is therefore a level playing field with respect to the approximate levels of

analytical sensitivity of these three methods. Defra and the FSA could therefore have confidence in the approximate levels of sensitivity of these three methods as applied as part of the UK Horse Meat Survey exercise, and therefore have assurance that the probability of detecting the horse DNA target was approximately the same between the three methods.

Acknowledgements

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