Inter-laboratory collaborative trial of real-time PCR method phase 2: materials and methods

2.1 Raw material handling

Beef, pork and horse meats were purchased from local suppliers as whole joints. These were stored at -20 °C prior to preparation. Excess fat was trimmed from the joints before mincing (grinding) using an electric mincer. The beef was minced first to avoid any contamination from the pork and horse meat. For each meat species, the first ~200 g of minced meat was discarded so that any residue in the mincing equipment would be removed with that initial portion. The mincing equipment was cleaned before and after mincing each meat type using a combination of methods.

After mechanical scrubbing of all parts of the mincer, the parts were soaked in a 10% bleach solution for 1 hour, sonicated in 10% bleach for 1 hour and then put through the dishwasher. Once out of the dishwasher all parts were sprayed with 1% Distel and wiped clean with paper towels. Swabs were taken from contact surfaces and analysed for the presence of each species DNA to ensure no cross-contamination occurred. The second ~200 g of minced meat for each species was retained as an environmental (negative) control for real-time PCR to ensure we had no contamination.

The meat mincing was located to a laboratory space that was several blocks removed from both the usual sample preparation facility at Fera and from the PCR laboratory. This was done to ensure the lowest possible risk of cross-contamination from the laboratory environment (where other meat sample handling is undertaken). An added benefit at the time of this project was to ensure sufficient physical working space when coronavirus social distance working mitigations were still in force at the Fera site.

Ingredients for the processed samples were tomato purée (Morrisons supermarket own-brand), cornflour (Sainsbury's supermarket own-brand) and gravy mix (Bisto gravy powder from ASDA supermarket). The gravy powder ingredients are listed as: potato starch, salt, wheat starch, colour (ammonia caramel), onion powder, inactive yeast powder. The risk of an incurred cross-contamination with target meat DNA of these non-meat ingredients (including the gravy mix) was considered to be negligible. The same precautions were undertaken within the laboratory environment to minimise cross-contamination from the meat preparation.

2.2 DNA Extraction and Authenticity Testing

Prior to the verification of the sample types for the collaborative trial (homogeneity testing), the PCR method was validated in-house according to the principles of current standard methods [7, 8]. The full validation is the subject of a separate report and not detailed further here.

Following mincing, each meat species DNA was extracted from each species using the commercially available DNeasy® Mericon Food Kit (Qiagen) which is a cetyltrimethylammonium bromide (CTAB) method. Minced meat (2 g) was homogenised in 10 ml lysis buffer and proteinase K by mechanical disruption in a vortex. The homogenised mixture was then incubated at 60 °C, centrifuged and the cell lysis supernatant was phase-separated with chloroform. DNA

was washed and precipitated in ethanol and eluted in 50 µl of elution buffer from the kit. The extraction method was also assessed in DNA fragmentation and a variety of modifications were trialled in the method. Modifications included differing lengths of incubation (30 or 60 minutes) and differing elution volumes (50 µl, 100 µl or 150 µl).

Because the input mass of each test portion of the meat was restricted for practical reasons (due to tube sizes) the eluates were individually measured on the spectrophotometer and the 260/280 nm and 260/230 nm ratios were noted. All individual extracts passed minimum performance criteria for quality and quantity and so were pooled after extractions to make one homogenous extract for each meat species.
Each meat species was also authenticated by Sanger sequencing using cytochrome B primers fwd: 5'-CCATCCAACATCTCAGCATGATGAA-3' and rev: 5'-CCCCTCAGAATGATATTTGTCCTCA-3'. Standard curves for calibration were made from raw unprocessed 100% w/w horse and pork meat against the background myostatin mammalian gene target.

Meat combination

The samples were prepared in the combinations described in Table 1, where the target percentage mixes of DNA relate to the w/w percentage of raw meats combined prior to extraction.

Table 1 combinations of target species DNA in matrix DNA with nominal w/w percentage of raw meats prior to extraction

Sample: Horse	Sample: Pork in raw beef	Sample: Pork in processed beef
Horse DNA in processed beef DNA (0.1%)	Pork DNA in raw beef DNA (0.1%)	Pork DNA in processed beef DNA (0.1%)
Horse DNA in processed beef DNA (0.5%)	Pork DNA in raw beef DNA (0.5%)	Pork DNA in processed beef DNA (0.5%)
Horse DNA in processed beef DNA (1%)	Pork DNA in raw beef DNA (1%)	Pork DNA in processed beef DNA (1%)
Horse DNA in processed beef DNA (3%)	Pork DNA in raw beef DNA (3%)	Pork DNA in processed beef DNA (3%)
Horse DNA in processed beef DNA (10%)	Pork DNA in raw beef DNA (10%)	Pork DNA in processed beef DNA (10%)

The final meat combinations of nominal targets 10% and 3% w/w were prepared first.
Homogeneity on the raw meat combinations was assessed on the DNA extracted from these w/w combinations and was quantified slightly higher than the intended target (see results). The higher w/w combinations were then processed, and homogeneity was re-assessed. Lower final meat combinations of 1%, 0.5% and 0.1% w/w were made from the 3% w/w mixture. This was to ensure that no biases would be carried over from the 3% combination to the lower % w/w combinations. The higher w/w combinations were then processed, and homogeneity was re-

assessed.

Processed sample preparation

Processed samples were prepared according to the following recipe taken directly from the Defra report [5]:

65% meat, 27% water, 1.25% tomato puree, 3% cornflour and 3% gravy mix

The 65% meat was the total meat content of the processed sample, itself comprising the corresponding percentage combinations of beef and horse or pork, defined in Table 1 above. The meat was added to the other ingredients at ambient temperature, stirred and gradually heated in a lidded vessel on a hot plate to a temperature of 72 °C for 15 minutes. The mixture was stirred occasionally during heating and then left to cool at ambient temperature prior to subsampling.

The lower % combinations for processed matrices were made from 3% raw meat and then cooked to give processed % w/w combinations at 1, 0.5 and 0.1%, i.e. 3% processed meat was not used to make lower % combinations.

Consumables procurement

Protein swabs and Real-time PCR Universal Mastermix (reagents Taq polymerase, dNTPs, buffer) were purchased from Fisher Scientific. DNeasy® Mericon Food Kits were purchased from Qiagen. Skirted tubes (0.5 ml), tube caps, centrifuge tubes (50 ml and 1.5 ml) were purchased from Starlab. Labels for sample tubes were purchased from Barcode Warehouse (to be compatible with the LIMS). Myostatin horse and pig assays were purchased from Eurofins (assay details in Report Appendix 1, sub-Appendix 1, Table A1, page 22).

Sufficient PCR consumables were purchased for both the internal validation of the methods and for the collaborative trial itself (where this means all sample preparation and verification/homogeneity testing plus consumables for the participants). Consumables to be distributed to the participants in the interlaboratory trial were aliquoted out in preparation for eventual dispatch.

PCR equipment

All assays and standard curves were validated on QuantStudio 6 Flex Real-time PCR systems (Applied Biosystems).

2.3 Recruitment of participating laboratories

Laboratories were recruited for the collaborative trial by invitation. The criteria for selection of laboratories to invite were whether they were UK official control laboratories, commercial laboratories in the UK and overseas that had a known track record in meat authenticity analysis, and the four laboratories that had taken part in the previous interlaboratory validation [5, 6]. All communications were sent through the Fapas® general email account (info@fapas.com), rather than any personal email account, to reduce the potential for the invitation to be caught in any junk mail filter. The use of a central email account also ensured consistency of communications and avoidance of any unintended bias.

Positive responses to the invitation were received from five UK official control laboratories and ten commercial or official control testing laboratories from eight countries. The laboratories that took part in the collaborative trial are listed in alphabetic order in Table 2. The order in Table 2 does

not correspond with the laboratory number in the results.

Table 2, laboratories that took part in the collaborative trial.

Laboratory name	Country
Campden BRI (Chipping Campden) Limited	UK
City of Edinburgh Council	UK
Eurofins Genomics Europe Applied Genomics GmbH	Germany
Fera Science Limited (Fera)	UK
Glasgow Scientific Services	UK
Hampshire Scientific Services	UK
Laboratorio de Salud Publica - Madrid	Spain
Minton Treharne and Davies Limited - Cardiff	UK
Premier Analytical Services	UK
Public Analyst's Laboratory - Cork	Ireland
Service de la consommation et des affaires veterinaires (SCAV)	Switzerland
SGS VIETNAM Ltd	Vietnam
Singapore Food Agency, National Centre for Food Science (Perahu Road)	Singapore
State General Laboratory	Cyprus
Tayside Scientific Services	UK

2.4 Collaborative trial design and logistics

The collaborative trial followed the randomised blind duplicate design [9], whereby each participating laboratory received 30 individual samples labelled PCR_01 to PCR_30 with no indication of what the sample number corresponded to. The 30 samples comprised 15 sample

types with one replicate each. The sample numbers corresponded to the sample types as presented in Table 3.

Table 3, sample identifiers and the corresponding sample type and nominal concentration of raw meats (% w/w) prior to extraction

Sample identifier	Sample type	Nominal level, %
PCR_01	Pork in raw beef	1
PCR_02	Horse in processed beef	1
PCR_03	Pork in processed beef	0.5
PCR_04	Pork in raw beef	0.5
PCR_05	Horse in processed beef	3
PCR_06	Horse in processed beef	1
PCR_07	Pork in processed beef	1
PCR_08	Horse in processed beef	3
PCR_09	Horse in processed beef	0.5
PCR_10	Pork in raw beef	3
PCR_11	Pork in processed beef	0.5
PCR_12	Horse in processed beef	0.5
PCR_13	Pork in processed beef	0.1
PCR_14	Pork in processed beef	0.1
PCR_15	Pork in raw beef	10
PCR_16	Pork in raw beef	3

Sample identifier	Sample type	Nominal level, %
PCR_17	Horse in processed beef	0.1
PCR_18	Horse in processed beef	0.1
PCR_19	Horse in processed beef	10
PCR_20	Pork in processed beef	10
PCR_21	Pork in processed beef	1
PCR_22	Pork in processed beef	3
PCR_23	Pork in raw beef	0.1
PCR_24	Pork in processed beef	10
PCR_25	Pork in processed beef	3
PCR_26	Horse in processed beef	10
PCR_27	Pork in raw beef	1
PCR_28	Pork in raw beef	0.1
PCR_29	Pork in raw beef	10
PCR_30	Pork in raw beef	0.5

In order to expedite results returns from the participating laboratories in the trial, the Fapas® database and secure website was set up to manage registrations, sample shipment and results entry. Use of the database negated any possibility of transcription errors from results returned by spreadsheet (for example). The Fapas® database is also designed to handle sample shipments, including addressing and linking directly to couriers, which has clear logistical advantages. The internal project reference for the database was set up as round MA0201, with individual sample references of PCR_01 to PCR_30 for the 30 samples being sent, as per Table 3.

All samples, together with essential consumables, were dispatched on 9 May 2022 to all 15 laboratories, with a results return deadline of 1 July 2022. The samples and consumables were shipped in insulated boxes with dry ice. Detailed instructions on sample handling and experimental protocol to be used were provided with the samples, including the primer sequences and a suggested layout of the PCR plates to encompass the number of determinations required.

These instructions are reproduced in Appendix 1. Raw data collected on spreadsheets were to be retained by the laboratories until requested.