Inter-laboratory collaborative trial of real-time PCR method phase 1: materials and methods

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.

DNA extraction and Authenticity

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.

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

Horse	Pork DNA in raw beef	Pork DNA 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). Lower final meat combinations of 1%, 0.5% and 0.1% w/w were made from the 3% w/w mixture, after checking yield, quality and homogeneity of 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 purée, 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.

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).