Practical modifications of the "ORBIT" screening system for fresh meat speciation

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Introduction: As a result of recent, well publicised, meat adulteration problems, an increasing number of UK processors are now seeking simple, reliable and cost-effective means of identifying meat species in their bulk, raw supplies. Although most reported cases have involved substitution of horse meat in frozen boneless boxed beef, accurate routine testing of raw processed material such as mechanically deboned meats (MDM) is also of potential interest. The classical serological tests are currently favoured by many processors with quality control facilities, ie. interfacial ring tests, Ouchterlony double diffusion or counter immunoelectrophoresis. None are performed, however, in a way which can be properly standardised without reference to a complicated protocol. Furthermore, because of variation in the responses of anti-species antisera, commercially available products must be checked against a wide range of meat species for cross-reactivity and to establish sensitivity of adulterant detection (eg. for ensuring the absence of horse meat in boxed beef). The introduction of one, simple and universally agreed method is now required. Recent applications of enzyme-linked immunosorbent assay (ELISA) for meat speciation (Jones, 1985) are promising but depend on "super-specificity" of antibody reagents, as for example, in the Checkmeat Kit (double-antibody sandwich ELISA, Patterson et al., 1984, 1985). However for routine monitoring this assay is expensive and considered still too complicated for unskilled users and also has a limited shelf-life. A recent modification of agar-gel immunodiffusion has been reported for verification of beef supplies in the USA (Mageau et al., 1984) and is known as the Overnight Rapid Bovine Identification Test (ORBIT). Even though this system is based on the slower "precipitin" type of antibody-antigen response it is cost-effective and most adaptable for field use: also the specific sheep anti-bovine antiserum is available in plentiful supply in a standard, stabilised form. The development of a similar series of "ORBIT" tests for verification of meats other than beef, ie. pig, horse and sheep/goat meats is now described. Laboratory trials have included assessment of model mince and MDM mixtures. Because the test is not yet muscle-specific, the circumstances for monitoring raw meats must be precisely defined.

Materials and methods:

Species-specific antisera: Antisera against bovine, porcine and equine species were raised in sheep with appropriate species albumin immunogens. Each primary dose comprised 5mg of albumin (Sigma, Fraction V products) emulsified in Freund's complete adjuvant (1ml) and sterile saline (1ml). Booster injections of 2mg albumin (made in the same manner and volume, except using Freund's incomplete adjuvant) were given at 28-40 day intervals. Antiserum against ovine species albumin was raised in a 12-month old steer by a similar programme. Trial bleedings (20-30ml) were taken 7-10 days after the second boost and antisera assessed for specificity by interfacial precipitin tests. In these, samples of each antiserum were overlaid with normal species sera (diluted 1:4 in 0.85% saline) in 6mm diameter pasteur pipettes. When the homologous response was rapid (ie. a white precipitate had formed at the junction of the two liquids during a 30m incubation at room temperature, RT) and heterologous responses were absent (no precipitate after 2h) the antisera were judged to be of sufficient titre for subsequent ORBIT preparation without further treatment. Several bleedings (up to 250ml) were then taken from each animal whilst the homologous responses remained high (up to two weeks after the booster injections). Three or four boosts were administered before terminal blood collection, which provided about one litre of antiserum in each case. The crude anti-ovine antiserum required partial purification and concentration to improve the intensity of its homologous response. Here the globulin fraction was precipitated with solid ammonium sulphate in two stages; at (1) 45% and (2) 40% saturation. The final centrifugation pellets were then redissolved in phosphate-buffered saline (using about one third of the starting serum volume), checked for response by interfacial ring tests and dialysed against the same buffer for 48h to remove remaining ammonium sulphate. The antisera were freeze-dried in bulk and stored in powder form. Each product was then reconstituted in water (80mg solid/ml) and filter-sterilised in 10-20 ml aliquots (Sartorius, Minisart NML, SM 16534) as required.

Reference meat extracts: Authentic species meat extracts were prepared from fresh, whole muscle tissue as follows: (1) Approximately 300g tissue was thoroughly comminuted to a fine slurry with 600ml of chilled water (in a food processor) allowed to stand for 1h at RT and centrifuged at 18-20,000 rpm for 30m. The supernatant was carefully withdrawn by pipette, to avoid floating fat, and used directly. (2) Other meat species fluids (drip) were pressed out of intact, frozen-thawed meat slices, diluted 1:1 with water and filter-sterilised as above. Since both types of extract contained many soluble meat proteins, as well as the residual blood components, each was checked by conventional Ouchterlony double diffusion tests (applying liquids direct to wells) to ensure that sufficient albumin had been extracted to develop the respective homologous, reference responses with overnight incubation at RT.

Preparation of stabilised reagent discs: The procedure of Mageau et al. (1984) was modified as follows: Blank absorbent discs (7mm diameter) were punched from Whatman No.3 paper and spread out on disposable petri dishes (Sterilin, 101VR20 ca. 40/dish). 20µl aliquots of liquid antiserum or meat extract were applied to the discs (prepared in batches of about 500) which were then allowed to absorb the liquid before air-drying at RT (1-2h) and freeze-drying overnight. Final stabilised discs were collected and stored in labelled air-tight vials at 4°C.

Preparation of agar plates: Petri dishes (as above) were filled with 15-20ml of clear, bubble-free molten agar prepared from the following: 3g agar (Oxoid, product no. L28), 1.7g NaCl, 0.1g sodium azide and 200ml water. The cooled, solidified plates, were mounted securely above one of the patterned templates (Fig. 1) as required.

Testing procedure for intact meat samples: The test procedure for verification of intact meat samples followed the main steps of the original ORBIT system as follows:
An agar plate was primed with reference meat discs (●) and antibody discs (★) of one of the species under test, using curved forceps to carefully lay each disc on the agar surface, so that all the template circles were fully covered when viewed directly from above. A small incision was then made in each meat sample (thawed if previously frozen) and two blank discs placed inside to absorb the tissue fluids. In general, sufficient drip exuded from

intact pieces of meat to saturate the discs in a short interval (10-15 sec). They were then gently removed with the forceps and deposited on parallel test positions (S). Meat slices were also prepared for fluid absorption; in this case 1cm thick pieces were placed in separate petri-dishes and several blank discs laid on the freshly exposed muscle surface. The meat was then covered with petri lids (in surface contact) for up to 60 sec or until the discs were visibly saturated. In some cases fresh meat slices required gentle heating (40°C for 1h) to increase fluid exudation, but high pH samples, eg. dark cutting beef, were too dry for this procedure without an initial freeze-thaw treatment or water extraction (as above). The loaded agar plates were then labelled, covered and sealed for overnight incubation at RT (ie. 20-25°C).

Testing procedure for minced meat mixtures and homogenates: Authentic whole muscles of known species, trimmed of most fat, were coarsely minced and 1kg lots comminuted by food processor. MDM samples (pork, beef, veal, lamb, venison and chicken) were generously donated by a local processor and each lot, weighing approximately 500g, was thoroughly mixed in the flexible plastic container before sub-sampling. Meat/water extracts were made for convenience in the following manner: 100g sub-samples of the original material or 100g (total weight) mixtures were massaged manually with 50ml water in strong, clear plastic bags for about 60 sec. After standing for 20-30 min, the massaging was repeated to ensure effective mixing. The mixtures of mince and MDM ranged from 3-50% of one species in another, eg. beef in pork, horse in beef. After this treatment most "extractions" gave a viscous slurry to which the blank paper discs were applied; some exuded sufficient liquid to saturate the discs immediately, however in other cases it was necessary to pick up a small amount of slurry on each disc and deposit them directly on the appropriate test positions. The loaded agar plates were then sealed for incubation at RT as before.

Interpretation of ORBIT results: Characteristic ORBIT patterns are given in Fig 2. All developed as visible white precipitin lines typically seen in conventional liquid-well immunodiffusion tests (Wier, 1977) when sample and antisera are applied in similar positions. The ORBIT tests were classified as positive or negative only when the appropriate species reference line had formed between the authentic species meat disc and testing antiserum disc. Thus an ORBIT response (Fig 2A) was recorded as truly positive when the precipitin lines opposite each sample fused completely with both ends of the reference line. When precipitin lines were formed between the sample and antibody disc with incomplete fusion (Fig. 2C) this response, termed partial identity, was not classified as a true positive.

The negative ORBIT result (Fig 2B) was indicated when only the straight reference line developed and sample lines were absent. Other non-immune or "false-positive" precipitations (Fig 2D) which may occur, but not observed in this trial, would also be classified as negative.

Results:

The crude anti-bovine antiserum gave intense precipitin responses against bovine serum albumin (BSA) solution (1mg/ml) and normal bovine serum (diluted 1:4) during the 7-14d period after boost of immunogen. Slaughter blood was collected 10d after the final injection for maximum response. Subsequent Ouchterlony well tests, using this liquid antiserum, gave the expected homologous lines of identity against beef "drip" after overnight incubation at RT (16-20h). Similar tests with the anti-porcine (anti-pig) and anti-equine (anti-horse) antisera also gave clear, homologous Ouchterlony results against appropriate species meat fluid. However, although the anti-ovine (anti-sheep/goat) reagent gave a good homologous response, lines were more diffuse and formed in close proximity to the antiserum well rather than at the preferred intermediate position as shown for the other species.

Verification of intact meat samples: Preliminary ORBIT trials using beef exudate as reference and anti-beef discs confirmed the findings of Mageau et al. (1984) and established a similar sensitive overnight verification test for intact beef samples with the prescribed sampling procedure. However, our modification was devised for laboratory trials using discs carrying the equivalent of 20µL of antiserum, half the amount used above. Reference beef discs carried sufficient albumin to form the homologous line and typical ORBIT arcs of complete identity (Fig 2A) were readily seen in all the tests on intact beef samples after the 16-20h incubation. A cross-reaction with one type of venison, from the red deer species Cervus elaphus, (wild and farmed in Scotland) was also observed. However the precipitin result gave a different pattern (Fig 2C) which was interpreted as partial identity for this venison because "spur" lines developed towards each (S) position. Cross-reactions were absent against the other species meats available, (ie. pig, horse, sheep, goat, chicken, turkey, rabbit and kangaroo), and various normal species sera (ie pig, horse, sheep, goat and rabbit), freeze-dried on discs for convenience.

The modified ORBIT-style tests set up to verify pig and horse meat were equally effective for whole meat samples using similar discs carrying 20µL of the crude anti-pig or anti-horse antisera and with appropriate species meat drip discs as reference. ORBIT arcs of complete identity developed in every case for the homologous species and cross-reactions of complete or partial identity were absent, even after prolonged incubation (7d). A similar ORBIT-style test for sheep and goat was also prepared. However, even after minor modification, its response was slower using the crude antisera, lines of identity taking more than 40h to develop. The purification and concentration was partially successful; precipitin lines, once developed, were much clearer but the incubation time required varied from 16-18h and no improvement was obtained after additional treatment of the host animal. The ORBIT arcs of complete identity did not differentiate between sheep and goat meat (as expected) but all other species tested including venison gave negative results after the 48h incubation time.

Analysis of meat mixtures: The simple massaging extraction method followed by ORBIT-disc test was an effective means of species verification for laboratory samples of minced meat (fat content up to 30%) and MDM (fat content up to 40%) supplied as single species products. Positive response lines were intense and clearly defined for verification of all the beef, pig and horse samples after overnight incubation, but sheep/goat verification had to be based on a fainter, more diffuse identity arc. The gel surface acted as an effective barrier to all but the soluble meat components and no interference around precipitin lines due to particles of muscle fibre, fat or connective tissue was observed. However, after prolonged incubation - 24 to 48h, the high concentration of soluble meat proteins gave a pronounced halo around some of the reference discs and sample points. After 16-20h

incubation, presence of the nominated test species, beef, pig or horse was confirmed in all the model minced meat mixtures above 10% (Table 1) regardless of fat content and beef or pig was confirmed in similar MDM mixtures. Although the lowest limit for sheep/goat detection was 15% at this time, most substitutions from 5-10% were successfully identified after a further 24h incubation. Careful examination of precipitin arcs was important with the 3-5% substitutions because it was possible to confuse certain negative, reference lines (which tended to curve in towards the antibody discs) with positive arcs when the sample line was present but very faint.

Discussion:

The ORBIT-disc tests described here for beef, pig, horse and sheep/goat have been developed to meet current demand for stricter routine quality control by the British meat processing industry and Law enforcement authorities. The original American ORBIT system of Mageau et al., (1984) demonstrated certain practical improvements for beef verification which were readily repeated by this study. It was also accurate, cost-effective and suitable for field use by inexperienced operators. However, the most useful attribute of ORBIT-style tests, such as in this laboratory's development, was that both antisera and reference species meat extracts were conveniently supplied in a very stable, standardised and ready-to-use form. This should reduce possible variation and error in test response by different antisera products and overcome the problem of providing similar authentic species meat extracts at short notice.

Trial kits, supplied with a simple pictorial guide and sufficient discs to carry out 25 duplicate tests for each species (beef, pig, horse and sheep/goat) have now been evaluated by 10 laboratories including representatives of the UK meat industry, public (County) analysts and other participants routinely involved with meat species identification. In this, no attempt was made to define conditions for meat sampling or preparation and the laboratories were free to test their own meat materials. Most evaluators returned favourable results and concluded that the system was suitable for application in an industial situation or by a practising analyst. In our laboratory trials, mixtures of common meats were analysed which bore some relevance to problems often encountered by the industry, such as determination of sensitivity levels in MDM.

The test, as now developed, is based on the well-known precipitin recognition of residual blood proteins in meat (see also Hayden, 1978; Doberstein & Greuel, 1982 and Swart & Wilks, 1982) and consequently suffers from several disadvantages which restrict its applications to well defined circumstances:

a) Closely related species eg. sheep and goat, horse and donkey, beef and buffalo cannot be differentiated because tests always result in responses of complete identity. Even with the use of antisera produced from closely related host animals (Weitz, 1952; Patterson et al., 1984) and subsequent absorption treatment of antisera to improve specificity such differentiation still requires a more sensitive "immunoassay", such as ELISA to identify unique species-specific epitopes and provide reliable positive or negative results.

b) Other unusual cross-reactions of economic importance may be encountered, for example the partial identity response for beef/venison (not reported by Mageau et al.) of our sheep anti-beef antisera. The intact meats could be distinguished but were not resolved in beef/venison mixtures. Modification of the beef test, to block interaction of antibodies recognising the venison albumin, was successful but the subsequent beef-only response was faint and diffuse. An improved response was not obtained using a concentrated IgG fraction from the absorbed antiserum.

c) The presence of residual blood proteins, whilst sufficiently sensitive to give verification results in most overnight tests, indicate the species meat only when the samples are in the form of whole pieces, a constraint which may not be practicable in all industrial circumstances. Under routine use the 5-7% level detected is probably sensitive enough to ensure the absence of unwanted species such as horse meat in frozen, boxed beef after 16-20h, but more thorough checking would require the extra 24h incubation period because of the natural variability of residual blood in this tissue. In fact, as sample extraction and test completion required only minimal effort we considered the time delay for low substitution tests or for verifying the absence of nominated species would be justified during a routine factory turnover (48h maximum). Furthermore, since the cost per test involved was also minimal (25-50p per duplicate test), more sample sites could be tested, if required.

d) The current test can only be applied to verify/identify species in raw meat and meat products; however, further generation of suitable antisera should accommodate similar tests for heated products.

It is hoped that, after further interlab testing, to have kits produced commercially containing all the necessary components at an acceptable cost for routine use to enable laboratories to carry out initial screening tests on their incoming raw meat supplies.

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Table 1. ORBIT-disc test results on minced meat mixtures (numbers indicate the % substitution of test species in the "Base" species:beef pig, horse or sheep)

"Base species mince	"ORBIT" species disc test: Beef	Pig	Horse	Sheep/Goat
BEEF	All tests for beef 'base' clearly visible (+ve) at 16-20h	10(++)[a],[b]5(++), 4(-)(-),3(-)(+)	10(++),7(++), 5(-)(-), 3(-)(-)	25(++),20(++),15(++), 10(-)(+),6(-)(+),5(-)(+), 4(-)(-)
PIG	10(++)[a], 6(+)(++)[b], 4(-)(-),3(-)(-)	All tests for pig 'base' clearly visible (+ve) at 16-20h	10(++), 7(++), 5(++),3(-)(+)	25(++),15(++),10(++), 7(++),10(-)(++),6(-)(++), 4(-)(+),5(-)(-),3(-)(-)
HORSE	10(++),7(++),5(++), 3(+)(++)	10(++),7(++), 5(+)(++)	All tests for horse 'base' clearly visible (+ve) at 16-20h	10(+)(++),7(+)(++), 5(+)(++),3(-)(+)
SHEEP	10(++),6(-)(++), 3(-)(+)	15(++),10(++),7(++), 6(+)(++),5(-)(-), 3(-)(-),3(-)(-)	10(++),7(++), 5(-)(+),3(-)(+)	Some tests for sheep 'base' not fully developed at 16-20h All +ve at 40-44h

(a) Results at 16-20h in first bracket
(b) Results at 40-44h in second bracket, shown when (a) was inconclusive.
++ Response positive; clear and well-defined arcs.
+ Response just visible, difficult to interpret.
- Response negative, no visible arcs.

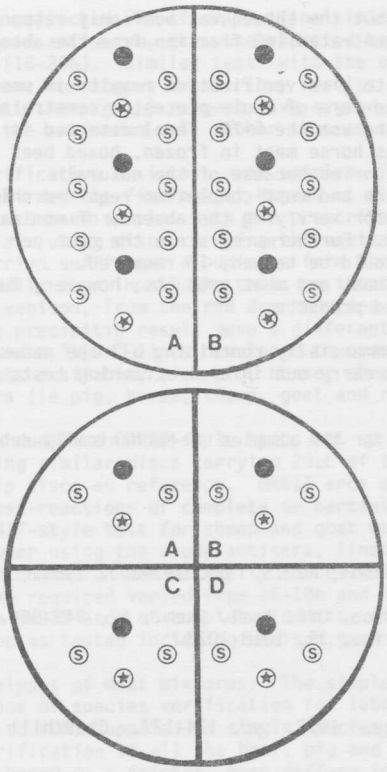

Fig. 1 Templates for ORBIT-disc application (for species A-D, as required)

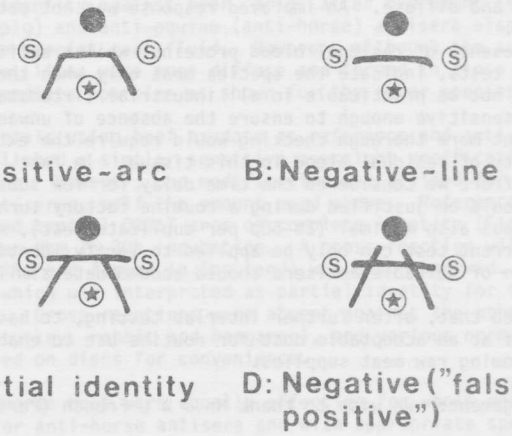

Fig. 2. Typical ORBIT-disc precipitin lines (visible in the agar gel)

KEY: ● Reference meat discs
⊛ Anti-species antibody discs
Ⓢ Loaded sample discs; duplicate tests applied as (Fig. 2) to obtain the characteristic arcs for positive identification