SPECIES IDENTIFICATION OF FRESH MEAT, HEAT-TREATED MEAT AND INTERNAL ORGANS USING ANTISERA TO THERMOSTABLE ANTIGENS

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1987
DECLARATION

This thesis is my original work and has not been presented for a degree in any other University

ERASTUS KIAMBI KANG'ETHE

This thesis has been submitted for examination with our approval as university supervisors


PROF. J.M. GATHUMA, B.V.Sc., M.Sc., Ph.D.
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Dedicated to my parents
SPECIES IDENTIFICATION OF FRESH MEAT, HEAT-TREATED MEAT AND INTERNAL ORGANS USING ANTISERA TO THERMOSTABLE ANTIGENS

Unequivocal identification of fresh meat and heat-treated meat is of special concern to food analysts and game conservation authorities. The food analyst has to provide proof of fraudulent substitution of more expensive meats with cheaper ones. Such adulteration represents unfair trading practices at either international or national level and is objectionable for reasons of culture, health or religion. The game conservation authorities need to differentiate meat of protected species from that of unprotected and domestic animals in their endeavour to curb poaching and protect endangered wildlife species.

It is imperative, therefore, that reliable analytical methods be developed to detect and quantify the adulteration in order to implement legislative measures necessary to achieve these objectives.

In this study, species identification of fresh meat and heat-treated meat was investigated using antisera to thermostable muscle antigens (TMA) obtained from the meat of 13 wild and 7 domestic animal species.
Antisera to TMA were raised in rabbits and goats. Unabsorbed rabbit antisera showed a wider range of cross-reactions than goat antisera. In immunodiffusion tests, rabbit and goat antisera showed two precipitin lines with homologous TMA and reacted also with serum, giving a reaction of identity with one of the two precipitin lines given by TMA of the same species. Thermostable muscle antigens of other species reacted with unabsorbed antisera to TMA, giving a reaction of identity with the precipitin line given by serum. This shows that serum contains a component of TMA which is identical or similar to one present in TMA of several species.

Absorption of rabbit antisera to TMA with copolymerized serum proteins rendered the rabbit antisera to TMA of oryx and camel species-specific, while the other antisera produced in rabbits retained cross-reactivities with TMA of several species. It was concluded that extracts of TMA contain related or similar antigenic components common to several animal species, especially within the bovidae, as shown by the wide range of reactivities of antisera produced in rabbits. It was virtually impossible to render these antisera species-specific by absorptions.

Although rabbit antisera to TMA lacked sufficient specificity to differentiate TMA of closely related species,
the antisera could be used to speciate TMA from distantly related species. In addition, antisera raised in rabbits to TMA of closely related species can be used to screen for the presence or absence of meat from bovidae and non-bovidae species. Rabbit antisera to TMA of topi, warthog and Grant's gazelle were proved to be useful in this regard.

The same absorption procedure rendered antisera produced in goats specific for all species, except the antisera to TMA of kongoni, topi, Thomson's gazelle and Grant's gazelle. Goat antiserum to TMA of kongoni reacted with the TMA of topi and wildebeest, while goat antiserum to TMA of topi reacted only with TMA of kongoni. Goat antisera to TMA of Thomson's gazelle and Grant's gazelle reacted with the TMA of both species. These results demonstrate the importance of selecting the animal species in which the antiserum is to be raised, in order to reduce the cross-reactions with antigens from closely related species.

Goat antisera also identified the species of origin of fresh meat using fresh meat antigens (FMA), and cooked meat using cooked meat antigens (CMA) in immunodiffusion tests. However, goat antiserum to TMA of cattle reacted with CMA and FMA of buffalo, thus failing to differentiate them from those of cattle. Cattle CMA and FMA could be differentiated from those of buffalo by using goat antiserum to TMA of buffalo.
The CMA and FMA of Thomson's gazelle and Grant's gazelle, - topi and kongoni, - pig and bushpig, - were not easily distinguishable using goat antisera to TMA of these species in immunodiffusion tests. While antisera to TMA produced in rabbits possessed limited capability to distinguish between species, goat antisera to TMA were capable either singly or in combination to identify all the FMA, CMA and TMA from the twenty animal species investigated in this study.

The results of immunodiffusion tests using TMA, CMA and FMA of a single species and goat antiserum to TMA, suggest that TMA are naturally occurring antigens of muscle tissue and are not novel antigens arising from heat treatment.

Identification of the species of origin of internal organs (liver, kidney and heart) was easily achieved using thermostable organ antigens (TOA) and goat antisera to TMA in immunodiffusion tests. The reactions of TMA, TOA with goat antiserum to TMA, revealed that there exist (i) a species-specific thermostable antigen common to muscles and organs, (ii) a species and striated muscle-specific antigen, found in skeletal and cardiac muscles and (iii) a species and tissue-specific antigen restricted to skeletal muscles.

Hydroxyproline was demonstrated in the TMA extracts, indicating the presence of gelatin in these preparations. Other protein contaminants were also shown to be present in
the TMA extracts. These contaminants inhibited the adsorption of the TMA to microtitre plates. The inhibitory effect of gelatin was demonstrated using commercial gelatin. Five milligrams of gelatin per millilitre resulted in complete inhibition of the adsorption of partially purified TMA (PTMA). Gelatin and the other contaminants are, therefore, undesirable in the enzyme immunoassay (EIA). A modification of the extraction procedure incorporating a partial purification step was adopted and yielded PTMA suitable for use in EIA.

The molecular weight of the PTMA, as determined using buffalo PTMA in gel filtration experiments, was calculated to be 210 kd.

In spite of the apparent high specificity of goat antisera in immunodiffusion tests, several antisera showed cross-reactions with PTMA of several species in quantitative EIA. However, goat antisera to TMA of buffalo, topi, pig, camel and horse were shown to be specific for these species. Nine other antisera showed various degrees of cross-reactions with 2 to 10 heterologous PTMA. Some of these reactions were nearly equal to those given by the homologous systems, thus failing to differentiate homologous from heterologous PTMA.

Goat antisera to TMA of cattle and eland cross-reacted with 13 and 15 heterologous PTMA respectively, giving reactions which did not allow unequivocal differentiation of
the homologous from the heterologous PTMA. In addition, the
goat antiserum to TMA of cattle gave only weak reactions with
PTMA of topi, pig, horse and Thomson's gazelle, which enabled
differentiation between the homologous and these heterologous
PTMA at $P \leq 0.05$. Similarly, goat antiserum to TMA of eland
gave weak reactions with PTMA of horse and cattle which
allowed differentiation between the PTMA of eland and those
of cattle and horse at $P \leq 0.05$.

In instances where a single antiserum failed to distinguish
between homologous and heterologous PTMA, species
identification could be achieved in enzyme immunoassay by
employing a number of goat antisera to TMA of several
species. Goat antisera to TMA provided reagents that were
capable of correctly identifying the PTMA of eighteen species
examined.

Because of their exquisite specificity, monoclonal
antibodies to TMA would be more suitable for species
identification than the polyclonal antisera used in this
study. However, it is conceivable that immunization of
additional animals of different species, especially within
the bovidae, would yield polyclonal antisera with sufficient
specificity for use in enzyme immunoassays.

Indirect EIA was also used to detect the presence of beef
in laboratory prepared beef/pork sausages. The test detected
beef at 5% level in pork sausages.
Milk powder commonly used as a binder in meat products did not give false positive reactions for beef in EIA, using goat antisera to TMA. Antisera to TMA can therefore be used to detect adulterants in fresh and heat-treated meat products containing milk proteins.

Detection of beef and pork in 90 commercially prepared beef and pork products from two factories were analysed using EIA. Fifty per cent of the pork products from both factories were found to contain beef, while 78.6% of the beef products from factory A and 6.3% from factory B, were found to contain pork. This may not be regarded as adulteration since specifications by the Kenya Bureau of Standards for fresh and heat-treated beef and pork products allow inclusion of flesh from other species, so long as the stipulated content of either beef or pork is maintained in the products. The specifications of the Kenya Bureau of Standards also state that the actual ingredients used in the manufacture of any food product must be shown on the attached label.

This study has shown that antisera to TMA produced in selected animal species possessed sufficient specificity to allow species identification of meat which had been subjected to heat-treatment which destroys the more abundant and easily identifiable heat-labile species-specific antigens. The same antisera also made possible the determination of species of origin of fresh meat as well as of internal organs such as kidney, liver and heart.
1. INTRODUCTION
Meat is the edible flesh of animals used as food after the completion of a series of biochemical and biophysical changes initiated in the muscles after the death of the animal. Animal meat is composed of muscle fibres held together by connective tissue through which blood vessels, nerves and fat cells are distributed. Proteins are the most abundant substances in animal tissues and are, without doubt, the most important constituents of meat.

Muscle proteins can be divided broadly into those which are soluble in water or dilute salt solutions (the sarcoplasmic proteins), those which are soluble in concentrated salt solutions (the myofibrillar proteins) and those which are insoluble in the latter, at least at low temperatures (proteins of the connective tissue and other formed substances) (Lawrie, 1979).

Muscles of all animal species contain similar proteins which perform similar biochemical functions. Interspecies differences in muscle composition are very small and differentiation of meat from different species using visual, histological and empirical chemical analysis is therefore almost impossible. Identification of the species of origin of fresh meat, therefore, presents a challenging task to the food analyst and the game conservation authorities whose duties involve prevention of poaching and the protection of endangered species.
A recent trend in the international meat trade is the selling of deboned, frozen and boxed meat. Under these circumstances inclusion of meat from species other than those specified on the label is not uncommon. Recently, there have been reports of substitution of beef with horse meat in the United Kingdom (Anon, 1982), and beef with kangaroo in Australian beef sold to United States (Whittaker et al., 1982). The food analyst is called upon to provide proof that such substitution has occurred and to quantify the level of adulteration. It is therefore, necessary that reliable analytical methods be established to identify the species of origin of meat in the interest of fair trading, either at the international level or simply for the protection of consumers' rights.

Kenya is endowed with a large and varied population of indigenous wild mammals most of which are herbivores. Poaching and substitution of domestic animal meat with wild animal meat is possible. The wildlife conservation authorities are faced with the task of differentiating meat of protected species from that of unprotected and domestic species. Identification of the species of origin of meat is of considerable importance in veterinary forensic medicine involving meats and regulatory control of animal products.

Identification of species of origin of heat-treated meats is a more difficult task because heating denatures most of
the species-specific epitopes in fresh meat. Heating meat at 45°C, results in denaturation of myofibrillar and sarcoplasmic proteins (Lawrie, 1979), while at 65°C the collagen, the abundant protein of connective tissue, forms a gel and dissolves in the meat juices (Bendall, 1946).

Identification of the species of origin of heat-treated meat and meat products depends on the identification of thermostable muscle proteins that withstand the processing temperature, yet retaining their antigenicity and species specificity. It is also conceivable that the heating of meat may result in the emergence of new thermostable antigens with species-specific epitopes for only heated meats. The existence of thermostable antigens would be of great importance in the identification of the species of origin of cooked or heat-treated meat, meat products as well as fresh meat.

The objectives of this project were:

(i) to demonstrate the existence of species-specific thermostable muscle antigens and use antisera to these antigens in identifying the species of origin of autoclaved, cooked and fresh meat.

(ii) to identify the species of origin of internal organs using antisera to thermostable antigens.
(iii) to develop an enzyme immunoassay procedure suitable for use in identifying the species of origin of autoclaved meats.

(iv) to evaluate the usefulness of the antisera to thermostable muscle antigens in identifying the species of origin of meat used in commercially prepared fresh and heat-treated meat products.
2. LITERATURE REVIEW
Identification of the species of origin of meats has been accomplished using electrophoresis (Nakkila and Linko, 1955; Scopes, 1968; Kaiser et al., 1980), isoenzyme analyses of electrophoresed muscle extracts (Dilworth and McKenzie, 1970; Slattery and Sinclair, 1983) and serological methods (Pinto, 1961; Kamiyama et al., 1978b; Kang'ethe et al., 1982).

2.1 RAW MEAT

2.1.1 Electrophoretic methods

The use of electrophoretic techniques for identifying the species of origin of raw meats depends mainly upon differences in their electric charge and consequently different migration rates in an electric field. These differences show up as, (i) the number of protein bands formed indicating differences in their physical properties as evidenced by different electric mobilities; and in (ii) the staining intensity of the respective bands denoting differences in their concentration.

Various electrophoretic techniques have been used for identifying the species of origin of raw meats. These include paper electrophoresis (Nakkila and Linko, 1955); starch-gel electrophoresis (Latner and Angel, 1957; Scopes, 1968), polyacrylamide gel electrophoresis (Cowie, 1968; Ebermann and Barna, 1972; Kaiser et al., 1980) and isoelectric focusing (Mackie, 1979; Verbeke, 1983).
Several researchers have shown that identification of the origin of raw meat can be achieved by the analysis of the electrophoretic patterns of serum proteins (Ashton, 1957; Latner and Angel, 1957), sarcoplasmic proteins (Conell, 1953; Nakkila and Linko, 1955; Giles, 1962; Scopes, 1968; Choi et al., 1970; Ebermann and Barna, 1972; Spell, 1974; Lundstrom, 1981; Abdalla et al., 1983), myosin (Champion et al., 1970; Seki et al., 1980), tropomyosin (Parsons et al., 1969), and whole muscle extracts (Cowie, 1968; Mattey et al., 1970; Hoyem and Thorson, 1970; Mackie, 1979; Kaiser et al., 1980).

Addition of non-meat proteins such as soya bean, egg white and milk proteins in meat products during processing is a common practice in many countries. These proteins have been shown not to interfere with species identification using electrophoretic methods since they give characteristic electrophoretic patterns which are different from those of meat proteins (Hofmann, 1974; Hofmann and Penny, 1971; 1973; Penny and Hofmann, 1971; Shiga, 1980; Kaiser et al., 1981).

The concentration of muscle proteins, especially the sarcoplasmic proteins, has been shown to vary not only between species but also within species, and even between muscles of the same animal (Giles, 1962; Lawrie, 1953). Such differences, if not taken into account, would erroneously suggest a sample to be from a different species, though it may be from the same species as the reference sample. The
effects of post-slaughter treatment such as conditioning, electrical stimulation and freezing on the number, mobility and the staining intensity of protein bands formed in an electrophoretic pattern, when compared to the patterns obtained from freshly slaughtered animals, is not well documented. Tiecco (1980) showed that electrophoretic patterns obtained from frozen beef had fewer bands than those of a freshly slaughtered animal. Abdallah et al. (1983) showed that electrophoretic patterns of sarcoplasmic proteins from frozen and fresh beef differed to the extent that species identification based on comparing the electrophoretic patterns of fresh with the frozen samples could not be achieved.

2.1.2 Isoenzymes

2.1.2.1 Lactate dehydrogenase (LDH)

Dilworth and McKenzie (1970) used the electrophoretic patterns of the LDH isoenzymes to distinguish between various wild animals. These authors found that the LDH isoenzyme patterns could not differentiate between deer and beef. Similar results were obtained by Munday et al. (1974) when they failed to differentiate between the closely related forrester kangaroo and the Bennet wallaby. In their work on closely related ruminant species (cattle, sheep, goat and buffalo meats) Slattery and Sinclair (1983) observed no
significant differences in the LDH isoenzyme patterns and it was therefore not possible to differentiate these species on the basis of LDH patterns alone.

2.1.2.2 Esterase

Dilworth and McKenzie (1970) and Slattery and Sinclair (1983) used esterase isoenzyme patterns in addition to LDH pattern to differentiate between related ruminant species. Heinert and Klinger (1980) and Slattery and Sinclair (1983) used electrophoretic patterns of muscle extracts together with the esterase isoenzyme patterns in order to differentiate between related species. Esterase isoenzyme patterns alone are not suitable for use in identifying the species of origin of raw meat.

2.1.2.3 Phosphogluconate dehydrogenase (PGD)

King and Kurth (1982) examined the PGD isoenzyme patterns of raw meat in isoelectric focused gels. They were able to differentiate between sheep and goat. However, the use of PGD isoenzyme patterns is limited since it is only a qualitative method. Phosphogluconate dehydrogenase isoenzymes vary in quantity from animal to animal and can therefore not be used for quantitative assays.
2.1.2.4 Phosphoglucomutase (PGM)

King and Kurth (1982) stained for PGM and found that the pattern of this isoenzyme in ruminant species was not affected by postmortem treatment such as electrical 'stimulation' and incubation for 24h at 30°C. They also found that the PGM of beef and kangaroo were different, although the kangaroo PGM was not staining intensely outside the isoelectric point range of beef PGM. This would prevent the use of PGM patterns in detecting kangaroo meat in beef mixtures.

2.1.2.5 Adenylate kinase (AK)

Staining for AK isoenzyme pattern in raw kangaroo and beef samples, King and Kurth (1982) found that the isoelectric point of kangaroo AK isoenzyme was lower than that of beef and stained intensely enough to permit detection of kangaroo meat in beef mixtures.

2.1.3 Serological methods

Serological techniques offer attractive alternatives to electrophoretic methods and isoenzyme analyses. Serological techniques rely upon:

(i) the production of antisera with adequate titre and sufficient species specificity for the test purposes, and

(ii) the efficient extraction of the antigen(s) from the test
sample with only minimum alterations of their immunogenicity and in sufficient non-varying quantities to allow quantitative analysis.

Proom (1943) showed that species specificity of an antiserum varies with the route of immunization and with the number of subsequent boosters. He observed that intramuscular and subcutaneous routes of immunization resulted in antisera with greater specificity than antisera produced by intravenous and intraperitoneal routes. Specificity was also shown to decrease with subsequent boosters.

Antisera to serum proteins have been used by Murakami et al. (1969), Shunmugam and Ranganathan (1972), Kamiyama et al. (1978a) and Manz (1980) in agar gel diffusion tests to detect the species of origin of fresh meat. Raising antibodies to serum proteins for the purpose of species identification is based on the fact that current slaughter techniques leave residual blood in the carcass; of the total blood volume, 1.5-2% remains in the musculature (Warriss, 1977).

Katsube and Imaizumi (1968) suggested that serum proteins retained in the muscles might be used to identify the species of origin of meat in precipitin reactions with antiserum to serum proteins. Kamiyama et al. (1978a) and Kang'ethe (1981), using antisera to purified serum albumin, found that the principal antigen(s) in each of the meat extracts of
cattle, goat, sheep, pig and horse detected by the antiserum shared epitopes with serum albumin.

Species-specific epitopes of serum albumin were investigated by Kamiyama (1977 a,b) using cattle, sheep and goat albumins. He observed that albumins cross-reacted with rabbit antisera to albumins. Sheep and goat albumins showed only 10% specificity. Kamiyama (1977 b) raised antisera to N- and C-terminal fragments of cattle, sheep and goat albumins cleaved by cyanogen bromide. Antisera to the C-fragment showed a higher degree of species specificity than the antisera raised to the N-terminal fragment. However, the use of antisera to N- and C-fragments did not offer any added advantage when compared with antisera to intact serum albumin in the identification of the species of origin of meat.

Kamiyama et al. (1978 b) detected pig meat adulterant of beef at the level of 0.2-0.5% using passive haemagglutination test, while Swarts and Wilks (1982), using antiserum to whole blood, were able to detect 5-20% adulteration of beef with kangaroo meat in immunodiffusion tests.

Antisera to whole muscle extracts have been used by Shunmugam and Ranganathan (1972) to differentiate between sheep and goat meat.

Isolation of muscle proteins to serve as antigens of choice in raising antisera for use in species identification has been attempted with some success by Furminger (1964),
Hayden (1977, 1979) and Schweiger et al. (1983). Furminger (1964) used antisera to myosins, but species identification was not possible because of the high degree of cross-reactions of the antisera. Hayden (1977), however, using antisera to chicken troponin, succeeded in detecting chicken meat in beef sausages at the level of 1-5% in immunodiffusion tests. Schweiger et al. (1983) used antiserum to troponin-T to differentiate between turkey and chicken meat. Hayden (1979) produced antisera to isolated myoglobins from horses, pigs and sheep and was able to detect 3% level of adulteration of ground beef with sheep and pig meats in immunodiffusion tests.

During the last two decades, there has been a considerable increase in the number of immunodiagnostic tests available. Immunofluorescence and radioimmunoassay have been used as the methods of choice where high sensitivity was required. Immunofluorescence is a time consuming procedure which can not be easily automated. It can, therefore, only be used conveniently for a small number of samples. In contrast, radioimmunoassay is suitable for large scale operations, but the short half-life of the reagents, the rather expensive equipment and the regulatory control of the isotopes tends to preclude the use of radioimmunoassay in small laboratories. These considerations promoted a search for detection labels for antibodies or antigens and led to
the use of enzymes and the development of enzyme immunoassay (EIA).

Enzyme immunossays are classified as heterogeneous or homogeneous assays. Homogeneous immunoassays are largely used for assaying small molecular weight substances, while heterogeneous enzyme immunoassays have found use in species identification of meat.

Enzyme linked immunosorbent assay (ELISA) was pioneered by Engvall and Perlmann (1971) and Van Wemeen and Schuurs (1971) based on the original suggestions of Miles and Hales (1968). Several modifications of EIA are now in use.

Patterson et al. (1984), and Jones and Patterson (1985) used the double antibody or 'sandwich' EIA to differentiate unprocessed beef, sheep, horse, kangaroo, pig, camel, buffalo and goat meats to a level of 1% adulteration in mixtures of two species. Jones and Patterson (1985) were able to detect a level of 0.5%.

Kangethe et al. (1982) used the indirect EIA to differentiate between beef, mutton, veal, goat, venison, pork and horse meat using antisera to bovine, sheep and horse serum albumin. Using this technique, they were able to detect 3% level of adulteration of beef with horse meat. Lower percentages of horse in beef (0.2-0.5%) made by mixing appropriate aliquots of pure species extracts gave significant results for horse meat down to 0.5%. Using a similar
technique, Whittaker et al. (1983) obtained similar results to those of Kang'ethe et al. (1982). A modification of indirect EIA where the antibody is incubated with varying amounts of antigen and the free antibody is separated, and then reacted with a sensitized plate was used by Kang'ethe (1981). In this work, it was not possible to quantify precisely the amount of horse meat added to ground beef in known mixtures. This was attributed to the variability in the content of residual blood in various muscles after slaughter. Griffiths and Billington (1984) found that this variation in the amount of blood left in muscles and its deterioration with time hindered accurate determination of the level of adulterant added, thus confirming the observations of Kang'ethe (1981).

2.2 **COOKED MEATS**

Identification of the species of origin of cooked or processed meats presents considerable obstacles to food analysts and forensic scientists because most of the species-specific proteins used in the identification of fresh meats are denatured by the process of heating.

2.2.1 **Isoenzyme analyses**

Differentiation of cooked meat by means of isoenzyme analysis in isoelectric focused gels was described by King (1984). Two isoenzymes namely, adenylate kinase and creatine kinase have been shown to be of some use in the identification of the species of origin of cooked meats.
2.2.1.1 Adenylate kinase (AK)

King (1984) found AK to be useful in the discrimination of possum, buffalo, cat, dog, horse, donkey, red and grey kangaroo meats from beef. The isoelectric points of the AKs' from these species were lower than that of beef. However, the activity of AK isoenzyme decreased in intensity in products heated at 105°C for 30 min. Because of the low recovery rate of AK activity in products heated to 120°C for 30 min, AK isoenzyme patterns are not suitable for use in differentiation of canned or meat products heated above 105°C for 30 min (King, 1984).

2.2.1.2 Creatine kinase (CK)

The CK isoenzyme patterns from beef, pig and sheep showed significant differences in their isoelectric points, thus enabling these species to be identified (King, 1984). Creatine kinase had a higher recovery rate than AK in products heated at 120°C for 30 min. This makes CK a more promising isoenzyme to use in the species identification of meat from canned products than AK.

Creatine kinase has been shown to exist as a dimer of two peptide chains of 41,000 daltons each (Bickerstaff and Price, 1978). Because of existing as a dimer, there is a possibility of a hybrid dimer formation which would complicate interpretation and may preclude identification if a sample
consisted of a complex mixture. Indeed, King (1984) observed such an hybrid dimer formation in mixtures of mutton and pork, mutton and beef, all in the ratio of 1/1.

2.2.2 **Histidine dipeptides**

The histidine dipeptides, carnosine (B-alanyl-L-histidine) and anserine (B-alanyl-L-1-methylhistidine) have long been known to be present in muscle tissue (Briscas and Fromageot, 1953). A third histidine dipeptide, ophidine or belanine (B-alanyl-L-3-methylhistidine) has also been shown to be present in muscle tissue (Rangeley and Lawrie, 1976, 1977; Harris and Milne, 1980, 1981). Carnegie et al. (1982, 1983) have shown pig muscle to contain a higher concentration of ophidine than muscles of kangaroo, cattle and horse. Ophidine has been shown to vary in concentration not only with age (Rangeley and Lawrie, 1976; Harris and Milne, 1981) but also within muscles of the same animal (Carnegie et al., 1982).

The anserine/carnosine ratio and the content of ophidine have been used by Tinbergen and Slump (1976) and Carnegie et al. (1982) to identify the species of origin of meat used in processed luncheon meats and tinned hams. Carnegie et al. (1982) used ophidine/anserine and carnosine/anserine ratios to differentiate between horse, kangaroo, beef and mutton when they were added to pork products in increasing amounts. However, differentiation based on these histidine dipeptide
ratios of other species such as buffalo, kangaroo and rabbit was not possible (Carnegie et al., 1982).

Ophidine is found only in the structure of actin and myosin (Johnson et al., 1967). It has been used in the determination of the lean meat content in meat products (Rangeley and Lawrie, 1976, 1977; Poulter and Lawrie, 1980a, b; Jones et al., 1982).

2.2.3 Fats

The curing process and other heating steps in the preparation of processed meat products denature proteins, while fats are resistant to these processing treatments. Fats have, therefore, been used for the identification of the species of origin of cooked meat products.

The pig is efficient in incorporating palmitic acid at position 2 of its triglyceride molecule (Christie and More, 1970; and Brockerhoff et al., 1966). This positional incorporation of palmitic acid has been shown to be species-specific and this allows the differentiation of lard from tallow (Verbeke and Brabander, 1979).

Horse meat can be differentiated from other meats by its linolenic acid content (Payne, 1971). Beef can be differentiated from chicken on the basis of oleic acid proportion in position 2 and the distribution of the stearic acid in the triglyceride fractions (Verbeke and Brabander 1979, 1980).
2.2.4 Serological Methods

Identification of cooked meats rests on the isolation of species-specific thermostable muscle antigens that withstand the processing conditions. At the same time, these antigens must not be lost through juices expressed during cooking or loose their immunogenicity.

Murakami et al. (1969) noticed a decrease in antigenicity of meat heated at various temperatures when tested against antisera raised to unheated meat extracts. Katsube and Imaizumi (1968) had earlier shown that antiserum to boiled serum reacts with raw meat giving better species specificity than antiserum to unheated serum. They also showed that antigens detected in boiled serum were present in raw meat extracts.

Antisera to urea extracts of heat denatured muscle proteins were used by Snell and Mentz (1969) to identify the origin of heat-denatured meats from cattle, pig and horse. Heating of meat at 120°C for 1 h prior to extraction of the antigens with 7M urea did not alter their antigenicity.

Koh (1978) improved the extraction procedure by incorporating 2-mercapto-ethanol in the extraction procedure for denatured muscle proteins from beef skeletal muscle and soya bean proteins. He showed that rabbit antisera to beef skeletal muscle extract reacted only with beef skeletal muscle and cardiac muscle extracts. Cooking of the meat
prior to extraction did not alter the precipitin reactions of the extracts and their homologous antisera. He further demonstrated that soya bean protein (Promine-D) extract did not cross-react with antisera to beef skeletal muscle. Manz (1983) utilized the antisera to urea/mercapto-ethanol extracts of kangaroo meat to detect the presence of kangaroo meat in sausages using enzyme-linked immunosorbent assay. He was able to detect the presence of kangaroo meat in commercial sausages and frankfurters.

Anon (1970) and Karpas et al. (1970) used antisera to heated IgG to determine the species of origin of meat used in the manufacture of sausages. Adulteration to the level of 35% was detected in mixtures containing 3 different meat types. Hirose and Osler (1967) and Henney and Ishizaka (1968) observed that heating of meat resulted in the emergence of new antigenic epitopes. Karpas et al. (1970) noticed an increase in cross-reactions arising from the emergence of these new antigens.

Milgrom and Witebsky (1962) described the presence of thermostable 'adrenal-specific antigens'. Milgrom et al. (1963) further showed that thermostable antigens from horse adrenal glands existed as two distinct antigens, one species-specific and the other cross-reacting with antisera to adrenal extracts from other species. Milgrom et al. (1964) studied the species-specificity of the adrenal
thermostable antigens and showed that antisera to these antigens could be used to identify the species of origin of meat in food products, decayed organ tissues and the species of origin of the food eaten by an animal by testing faeces or urine of an animal fed with those samples. Andersen (1975, 1977a, b) and Hayden (1981) confirmed the results of Milgrom and Witebsky (1962) by using antisera to adrenal thermostable antigens in the identification of the species of origin of cooked meats. Hayden (1981) was able to detect 5-10% adulteration of cooked beef sausages with horse meat in immunodiffusion tests.

Monoclonal antibodies are superior to polyclonal antibodies because they react with single epitopes and are therefore more specific. Murine monoclonal antibodies to heated (70°C) meat from cattle and kangaroo were used by Goerlich and Greuel (1986) in ELISA to differentiate between heated and fresh cattle and kangaroo meats.

The state-of-the-art of meat species identification, is that fresh meat has been easily identified using physico-chemical and immunological methods. Identification of heat-treated or cooked meat has not been successful to the same degree employing the same methods used for fresh meat. The existence of thermostable muscle antigens would simplify species identification, as antisera to these antigens could be used to speciate heat-treated as well as fresh meats.
3. MATERIALS AND METHODS
3.1 ANTIGEN EXTRACTION PROCEDURE

Extracts of meat samples were prepared from 13 wild and 7 domestic species, namely: buffalo (Syncerus caffer), bushbuck (Tragelaphus scriptus), bushpig (Potamochoerus porcus), eland (Taurotragus oryx), Grant’s gazelle (Gazella grantii), impala (Aepyceros melampus), kongoni (Alcelaphus buselaphus cokii), oryx (Oryx gazella beisa), Thomson’s gazelle (Gazella thomsoni), topi (Damaliscus lunatus topi), warthog (Phacochoerus aethiopicus), waterbuck (Kobus spp), wildebeest (Connochaetes taurinus), cattle (Bos indicus), dog (Canis l. familiaris), goat (Capra aegagrus hircus), pig (Sus s. domestica), camel (Camelus dromedarius), horse (Equus p. caballus) and sheep (Ovis ammon aries).

Figures 1A, 1B, 1C are flow charts showing the evolutionary relationships of the animals studied.

The method of Milgrom and Witebsky (1962) of extracting adrenal thermostable antigens (BE = boiled ethanol insoluble antigens) was adopted for the extraction of thermostable muscle antigens (TMA), fresh meat antigens (FMA) and cooked meat antigens (CMA).

3.1.1 Fresh meat antigens (FMA)

Meat samples were dissected free of external fat and connective tissue, minced and homogenized in saline in a ratio of 2/1 (w/v). The homogenate was sonicated in an ice
bath at 300 W for 10 min. using a Braunsonic 1510 instrument (B. Braun, Melsungen A.G., Melsungen, W. Germany) with one minute bursts. The sonicated homogenate was centrifuged at 2,000 xg and the supernatant used as the source of FMA (Fig. 2).

3.1.2 Cooked meat antigens (CMA)

The samples were treated as in the FMA extraction procedure, except that the sonicated homogenate was placed in a boiling waterbath for 1 h, and rehomogenized. The volume of fluid lost was replaced by adding saline. The rehomogenized samples were centrifuged at 2,000 xg and the supernatant was filtered through a Whatman filter paper number 3 to remove fat. The filtrate was used as the CMA (Fig. 2).

3.1.3 Thermostable muscle antigens (TMA)

The samples were treated as in the extraction of CMA but further processed as follows: the filtrate was autoclaved at 121°C for 30 min. After cooling the filtrate was centrifuged at 86,000 xg for 30 min. The TMA were precipitated from the supernatant by the addition of 3 volumes of absolute ethanol and left overnight at room temperature. After centrifugation at 2,000 xg, the remaining ethanol was evaporated from the precipitate in a Rotavapour(R) (Buchi, Flawil, Switzerland). The dried precipitate was redissolved
Figure 1A: A simplified chart of the zoological relationship of the bovidae animals studied.
Fig. 1B: Flow chart of the zoological relationship of the non-bovidae animals studied
Flow chart of the zoological relationship of the non-bovidae animals studied

Fig. 1C : Flow chart of the zoological relationship of the non-bovidae animals studied
Meat samples, minced homogenized and sonicated

- centrifuged
- sediment discarded
- supernatant (FMA)

Heated in boiling waterbath, rehomogenized and centrifuged

- sediment discarded

Supernatant filtered, filtrate

- cooked meat antigens (CMA)

Autoclaved, centrifuged

- sediment discarded

Supernatant, precipitated with ethanol, centrifuged

- supernatant discarded

Precipitate dried, redissolved in saline and centrifuged

- sediment discarded

Supernatant (TMA)

Figure 2: Flow chart showing the extraction of FMA, CMA and TMA
in a small volume of saline and centrifuged at 86,000 xg. The supernatant was used as the TMA (Fig. 2).

All the antigens (FMA, CMA and TMA) were stored at -20°C after addition of sodium azide to 0.1%.

3.1.4 Thermostable Organ Antigens (TOA)

Thermostable organ antigens from the liver, heart and kidney of cattle, pig, kongoni and Thomson's gazelle were extracted using the method described for TMA (section 3.1.3.).

3.2 PRODUCTION OF ANTISERA

3.2.1 Production of antisera to TMA

Antisera to TMA were raised in rabbits and goats. Two New Zealand white rabbits and one goat, or sheep in case of antiserum to TMA of goat, were immunized with each TMA as follows: one millilitre of the TMA solution was mixed with 2 ml of Freund's complete adjuvant until a stable emulsion was formed. The animals were injected with the emulsion intramuscularly and subcutaneously at multiple sites. Booster doses were given after every 2 weeks, substituting Freund's complete adjuvant with Freund's incomplete adjuvant. The first bleeding was carried out 4 weeks after the primary immunization and thereafter every other week for 11 months. Serum was stored at -20°C after the addition of sodium azide to 0.1%. The animals used for immunization are listed in Table 1.
3.2.2 Production of rabbit antiserum to Goat IgG

Normal sera from 10 goats were pooled and the globulins precipitated using saturated ammonium sulphate solution, pH 7.4, according to the method of Fey et al. (1976).

The globulin fraction was dialysed against several changes of phosphate buffered saline (PBS) for 3 days and 0.02 M phosphate buffer, pH 8.0, for 1 day. The dialysed fraction was passed through a diethyl-aminoethyl cellulose column (BioRad Laboratories, Richmond, California, USA), equilibrated with 0.02M phosphate buffer, pH 8.0. The IgG fraction was eluted using 0.02M phosphate buffer, pH 8.0, and concentrated using a Diaflo ultrafilter with a cut off point of 30,000 daltons (Amicon, Massachusetts, USA). The fraction was shown to contain pure IgG by immunoelectrophoresis using rabbit anti-goat serum. The IgG fraction was used to immunize rabbits. The procedure for immunization was the same as that described in section 3.2.1.

3.3 ABSORPTION OF THE ANTISERA

3.3.1 Absorption of goat and rabbit antisera to TMA for use in immunodiffusion tests

Goat and rabbit antisera to TMA were absorbed to remove cross-reacting antibodies using copolymerized serum proteins, according to the ethylchloroformate method of Avrameas and Ternynck (1962). Eighty ml of pooled serum from the 20
Table 1: Experimental animals used for the production of antisera to TMA

<table>
<thead>
<tr>
<th>Antigen used (TMA)</th>
<th>Identification number of rabbit used</th>
<th>Identification number of goat or sheep used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>305 and LY-694</td>
<td>340</td>
</tr>
<tr>
<td>Bushbuck</td>
<td>LY-628 and LY-629</td>
<td>831</td>
</tr>
<tr>
<td>Bushpig</td>
<td>885b</td>
<td>892</td>
</tr>
<tr>
<td>Camel</td>
<td>886</td>
<td>817</td>
</tr>
<tr>
<td>Cattle</td>
<td>302 and 307</td>
<td>343</td>
</tr>
<tr>
<td>Dog</td>
<td>882</td>
<td>885</td>
</tr>
<tr>
<td>Eland</td>
<td>308 and LY-879</td>
<td>346</td>
</tr>
<tr>
<td>Goat</td>
<td>306 and LY-696</td>
<td>345</td>
</tr>
<tr>
<td>Grant's gazelle</td>
<td>313 and LY-632</td>
<td>344</td>
</tr>
<tr>
<td>Horse</td>
<td>881(b)</td>
<td>897</td>
</tr>
<tr>
<td>Impala</td>
<td>312 and LY-881</td>
<td>342</td>
</tr>
<tr>
<td>Kongoni</td>
<td>127, 314 and 317</td>
<td>826</td>
</tr>
<tr>
<td>Oryx</td>
<td>310 and LY-880</td>
<td>856</td>
</tr>
<tr>
<td>Pig</td>
<td>883</td>
<td>863</td>
</tr>
<tr>
<td>Sheep</td>
<td>LY-632 and LY-636</td>
<td>841</td>
</tr>
<tr>
<td>Thomson's gazelle</td>
<td>315 and 316</td>
<td>347</td>
</tr>
<tr>
<td>Topi</td>
<td>303 and LY-690</td>
<td>829</td>
</tr>
<tr>
<td>Warthog</td>
<td>884</td>
<td>869</td>
</tr>
<tr>
<td>Waterbuck</td>
<td>LY-633 and LY-634</td>
<td>832</td>
</tr>
<tr>
<td>Wildebeest</td>
<td>LY-693</td>
<td>827</td>
</tr>
<tr>
<td>Goat IgG</td>
<td>887, 889 and 892(b)</td>
<td></td>
</tr>
</tbody>
</table>
species were mixed with an equal volume of 0.2M acetate buffer, pH 5.0, while stirring. The pH of the mixture was adjusted to pH 5.0. Ethylchloroformate was added dropwise while stirring in amounts of 0.6 ml per 10 ml of serum. The stirring was continued for 15 min. while the pH was maintained between 4.5 and 5.0 by the addition of 1M NaOH. The mixture was allowed to stand for 1 h at room temperature. The gel formed was homogenized using a tissue grinder. The finely ground homogenate was washed 3 times in saline. After the final wash, the immunosorbent was suspended in saline and sodium azide added to 0.1%.

The antiserum was mixed with the insoluble immunosorbent and left on an end-over-end mixer, overnight at 4°C. The antiserum was separated by centrifugation at 2,000 xg for 15 min at 4°C. The absorbed antiserum was tested for cross-reactivity in immunodiffusion tests. If the antiserum was found to be specific, it was stored at 4°C after addition of sodium azide to 0.1%. If the antiserum was found to cross-react with heterologous TMA, it was further absorbed using the same immunosorbent after regeneration until maximum specificity was achieved. The immunosorbents were regenerated as detailed in section 3.3.3.
3.3.2 Absorption of goat antisera to TMA for use in the enzyme immunoassay (EIA)

The goat antisera to TMA absorbed with copolymerized serum proteins according to the method of Avrameas and Ternynck (1962) described in Section 3.3.1. were tested for specificity in an enzyme immunoassay (Section 3.5.9). The antisera were absorbed using the appropriate cross-reacting and partially purified thermostable muscle antigens (see Section 3.5.1.) coupled to cyanogen bromide activated Sepharose 4B, and further absorbed with saline extracts of raw meats from wildebeest and warthog copolymerized by the same method.

After absorptions, the antisera were tested for cross-reactions in EIA, and stored at 4°C after adding sodium azide to 0.1%.

3.3.3 Regeneration of immunosorbents

The immunosorbents were regenerated by washing 3 times with PBS and once with normal saline. The adsorbed antibodies were eluted with 0.2M glycine-HCl buffer, pH 2.5. The immunosorbents were washed with 0.1M Tris-HCl buffer, pH 8.5, containing 0.5M NaCl. After this final wash, they were suspended in PBS and stored at 4°C after addition of sodium azide to 0.1%.
3.4 IMMUNODIFFUSION TESTS

The microtechnique of Ouchterlony's double diffusion test as described by Crowle (1973) was used with slight modifications. One percent (w/v) purified Oxoid agar (Oxoid Ltd., England) in PBS was used as the medium for double diffusion. Sodium azide was added to 0.1%. Three ml of the molten agar was poured onto a microscope slide (75 x 25 mm) on a levelled table to give a depth of approximately 2-3 mm. Wells with a diameter of 4 mm and placed 5 mm apart were punched in a hexagonal pattern. The central well was filled with antiserum (absorbed or unabsorbed rabbit or goat antisera to TMA) while the peripheral wells were filled with the antigen solutions (TMA, CMA, FMA or TOA). The slides were incubated in a humid chamber and diffusion allowed to proceed for 12 - 48 h at room temperature.

The slides were rinsed with distilled water and pressed overnight using absorbent paper. They were then washed in 3% (w/v) trisodium citrate buffer, pH 8.5, for 12 h. The slides were rinsed with distilled water, pressed, rinsed and air dried at room temperature. The slides were stained with either Ponceau 'S' or Coomassie Brilliant Blue protein stains and destained until the background became sufficiently clear (Axelsen, 1973).
3.5 **ENZYME IMMUNOASSAY (EIA)**

3.5.1 **Preparation of partially purified thermostable muscle antigens (PTMA)**

Partially purified TMA (PTMA) was obtained from meat samples using a modification of the method of Milgrom and Witebsky (1962). Briefly, the following modifications were adopted: The sonicated homogenate was centrifuged at 2,000 xg, filtered, centrifuged at 86,000 xg and the supernatant autoclaved at 121°C for 30 min. After this step, other steps were similar to those used in extraction of TMA according to the method of Milgrom and Witebsky (1962) (see Section 3.1.3). After obtaining TMA, the TMA were concentrated on a Diaflo ultrafilter cell with a PM 30 filter. The concentrated TMA was fractionated through either Sephadex G-200 or G-75 column (height 94.5 or 35.5 cm respectively). Fractions of 7 ml were eluted using PBS. The antigen content of each fraction was determined by EIA (section 3.5.9) on the basis of optical density (OD) at 410nm. The tubes containing the antigen(s) were pooled and concentrated by ultrafiltration. Figure 3 is a flow chart showing the extraction procedure for the PTMA.

3.5.2 **Determination of the protein content of the PTMA**

The protein content of each PTMA was estimated using the Folin-phenol method of Lowry et al. (1951), using bovine serum albumin as the standard.
Meat samples, minced
homogenized, sonicated
centrifuged

- sediment discarded

supernatant, centrifuged

- sediment discarded

supernatant, autoclaved,
rehomogenized and centrifuged

- sediment discarded

supernatant, precipitated
and centrifuged

- supernatant discarded

sediment, dried
redissolved and centrifuged

- sediment discarded

supernatant, concentrated
on Diaflo PM 30

- filtrate discarded

concentrate, fractionated
on G-200

fractions tested for
antigen activity on EIA

- non-antigenic fractions
discarded

antigenic fractions
pooled, concentrated
on Diaflo PM 30

- Filtrate discarded

concentrate used
as PTMA

Figure 3: Flow chart showing the extraction of PTMA
3.5.3 Preparation of rabbit anti-goat IgG-glucose oxidase conjugate

The IgG fraction of rabbit anti-goat IgG serum was prepared as described in section 3.2.2, and conjugated to glucose oxidase (Sigma Co. Ltd) according to the metaperiodate method of Wilson and Nakane (1978) with slight modifications. The procedure adopted was as follows: Seventy six milligrams of glucose oxidase was dissolved in 9 ml of distilled water and oxidized with 1 ml of 0.1M sodium metaperiodate solution. The mixture was stirred in the dark for 30 min. at room temperature. The oxidized enzyme was dialysed against cold 1 mM acetate buffer, pH 4.4, containing 0.1 ml of ethylene glycol for 10 min, after which the dialysis was continued in 1 mM acetate buffer, pH 4.4, without ethylene glycol for 1 h with 4 changes of the buffer.

The enzyme was mixed with IgG fraction from rabbit anti-goat IgG serum (76 mg total protein) dissolved in 0.5 M carbonate buffer, pH 9.0, resulting in a molar ratio of 1/1. The pH was adjusted to 9.0 using solid sodium carbonate. The enzyme IgG mixture was left stirring at room temperature for 2 h, the pH was later adjusted to between 7.5 and 7.8 and left at 4°C overnight. Twenty milligrams of glycine were added to the mixture to block remaining aldehyde groups. Stirring was continued at room temperature for 1 h, and the pH finally adjusted to between 7.4 and 7.8 using 1 M HCl.
After clarification by centrifugation at 2,000 xg for 10 min at 4°C, the conjugate was filtered through 0.45 μm filter (Sartorius, Gottingen, W. Germany) and stored at -20°C after the addition of an equal volume of glycerol.

3.5.4 Choice of coating buffers

Three buffers were tested in EIA (section 3.5.10) for their efficacy in promoting adsorption of the PTMA onto the microtitre plates (Dynatech, M129A, flat bottomed). Buffalo PTMA was used in this assay. Buffalo PTMA was diluted in, 0.05 M carbonate-bicarbonate buffer, pH 9.6, 1:100 PBS + 2% polyethylene glycol 6000 (PEG) + 0.5 M NaCl and in 1:100 PBS + 2% PEG. The plates were incubated overnight in a humid chamber at room temperature. Other steps of EIA were as detailed in section 3.5.9.

3.5.5 Optimisation of PTMA concentration for use in enzyme immunoassay

Each PTMA was diluted 1/2 to 1/2048 in the coating buffer (0.05 M carbonate-bicarbonate, pH 9.6). Each dilution coated 1 column of the wells of a microtitre plate applying 100 μl/well. Using appropriate serum and conjugate dilutions, the optimal antigen dilution for coating the plates was determined by EIA (section 3.5.9).
3.5.6 Determination of optimal antiserum and conjugate dilutions

A microtitre plate was coated with 100 µl/well of the optimal dilution of each PTMA as determined in section 3.5.6. Optimum working dilutions of the antiserum and conjugate for each PTMA were determined by checker-board titrations.

Antigen, antiserum, conjugate and substrate controls were included every time.

3.5.7 Assay for hydroxyproline

Hydroxyproline was assayed for in the TMA, PTMA and in the Sephadex G-75 and G-200 fractions according to the method of Blumenkrantz and Asboe-Hansen (1975).

Five mls of each sample were hydrolysed in 6 M HCl at 120°C overnight. The hydrolysed samples were dried in a Rotavapour(R) and reconstituted to 2 ml using deionized water. To 1 ml of the reconstituted sample was added 1.5 ml of 1/10 borate-alanine buffer and 0.6 ml of 0.2 M chloramine-T solution. The samples were incubated at room temperature for 20 min to allow oxidation. After oxidation, 2 ml of 3.6 M sodium thiosulphate solution was added and the solution saturated with sodium chloride. Three millilitres of toluene were added and the samples placed in a boiling waterbath for 20 min. After cooling, the samples were thoroughly shaken for 15 min and centrifuged at 500 xg for 10 min. The organic phase was separated and 1 ml of the organic
phase was mixed with 0.4 ml of Ehrlich's reagent. The samples were allowed to stand for 30 min at room temperature for colour development. The colour intensity was measured at 565 nm using a Beckman Spectrophotometer, Model 25.

3.5.8 The effect of gelatin on the adsorption of PTMA to microtitre plates

Buffalo PTMA was diluted in the coating buffer to contain 42.7 µg/ml, and mixed with 5mg, 500ug and 5ug of commercial gelatin (Matheson Coleman and Bell, Los Angeles, USA). The mixtures were used to coat microtitre plates. The effect of gelatin on the adsorption of the PTMA was determined by EIA using appropriate dilutions of goat antiserum to TMA of buffalo and the rabbit anti-goat IgG-glucose oxidase conjugate. The effect of gelatin in reducing the adsorption of the PTMA was expressed as a percentage inhibition using the equation below:

\[
\% \text{ inhibition} = \frac{OD_z - OD_s}{OD_z} \times 100
\]

where \( OD_z \) = absorbance at 410 nm of buffalo PTMA without gelatin (0% inhibition)

\( OD_s \) = absorbance at 410 nm of buffalo PTMA plus gelatin at various concentrations
3.5.9 Enzyme Immunoassay for species identification of PTMA

The PTMA were initially diluted to contain the protein concentration which had been found to be optimal for each species. The PTMA were then diluted serially in double dilutions in the coating buffer and 100 µl/well of each PTMA dilution was used to coat duplicate wells of the microtitre plates. The plates were incubated in a humid chamber at room temperature overnight. The plates were tapped dry with absorbent paper and washed 5 times at 5 min intervals using the washing solution (PBS + 0.05% Tween 80). The plates were tapped dry, and 100 µl/well of the antiserum diluent (see Appendix 6.2) containing 5% normal rabbit serum was added, and the plates incubated at 37°C for 1 h. The plates were washed as described above, and 100 µl/well of the appropriate antiserum dilution added. The antiserum was diluted in the antiserum diluent (see Appendix 6.2). After incubation at 37°C for 1 h, the plates were washed and 100 µl/well of the appropriate dilution of the rabbit anti-goat IgG glucose oxidase conjugate added. The conjugate diluent was similar to the antiserum diluent except that 0.5% Tween 80 was used instead of 0.1% Tween 80 (see Appendix 6.3). The plates were incubated at 37°C for 1 h, washed, tapped dry and 100 µl/well of the substrate solution (see Appendix 6.4.1) added. The plates were incubated at room temperature for 1 h and the absorbance read at 410 nm using an ELISA Minireader (Dynatech Co. Ltd., Santa Monica, California).
3.5.10 Detection of the species of origin of heated meats in known mixtures

Beef was mixed with buffalo, goat with impala and pork with warthog meat at 1% and 10% levels. The PTMA from these mixtures were extracted according to the procedure described in section 3.5.1. Detection of buffalo, impala and warthog in beef, goat and pork respectively was done in EIA using appropriate goat antisera to TMA.

3.6 MOLECULAR WEIGHT DETERMINATION OF THE PTMA BY GEL FILTRATION

Determination of the molecular weight of the PTMA was carried out on a Sephadex G-200 column by the method of Andrews (1964).

The column was packed using the method described by Hudson and Hay (1980). The column height was 94.2 cm and the internal diameter was 2.6 cm. After equilibration with PBS, the column was calibrated using the following protein markers.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Molecular weight (daltons)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue dextran</td>
<td>2,000,000</td>
</tr>
<tr>
<td>Purified IgG</td>
<td>150,000</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>67,000</td>
</tr>
</tbody>
</table>
The calibration was repeated twice and the average void and elution volumes of the protein standards were determined. The Kav of each marker was calculated according to the following formula: ("Gel and filtration": Theory and Practice" Pharmacia Fine chemicals, Uppsala, Sweden).

\[
Kav = \frac{Ve - Vo}{vt - Vo}
\]

where

- \(Ve\) = void volume (elution volume of blue dextran)
- \(Vo\) = elution volume of the other protein marker
- \(Vt\) = total bed volume
- \(Kav\) = the fraction of the stationary gel volume which is available for diffusion of a given solute

The Kavs were plotted on a semi-logarithmic graph paper against their corresponding molecular weights. The best line which joins the points on the graph paper was drawn to give the molecular weight standard curve using the least squares method.

Three ml of the concentrated buffalo PTMA were applied to the calibrated Sephadex G-200 column. Buffalo PTMA was detected in the eluted fractions by EIA (section 3.5.9). Each eluted fraction was diluted 1/2 in the coating buffer and 100 ul was used to coat two wells of a microtitre plate. The other steps of EIA were as described in section 3.5.9.

The elution volume and the Kav of the PTMA was calculated. From the standard curve, the molecular weight of the PTMA was determined.
3.7 DETECTION OF BEEF AND PORK IN PORK AND BEEF MEAT PRODUCTS

Processed beef and pork products such as sausages, beef burgers, cooked ham, luncheon meat and canned corned beef were purchased from the retail outlets of two leading Kenyan manufacturing firms in Nairobi. Ninety samples bearing different production and batch dates were purchased over a period of 2 months.

3.7.1 Extraction of antigens from meat products

After the removal of the casings and other wrappings, the products were each homogenized in PBS in the ratio 2/1 (w/v). The homogenates were centrifuged at 4,000 xg for 30 min and the supernatant filtered through Whatman filter paper no. 3. The filtrate was stored at 4°C after adding sodium azide to 0.1%.

3.7.2 Preparation of pork and beef sausages and pork sausages containing various percentages of beef

Fresh beef and pork sausages and pork sausages containing 1, 5 and 10% beef were made in 1 kg lots in the laboratory. Table 2 shows the recipe for the preparations. Five percent milk powder was used in the sausage preparation.
Table 2: Recipe for the preparation of beef and pork sausages

<table>
<thead>
<tr>
<th>Constituents</th>
<th>Pork sausages containing beef</th>
<th>Beef sausages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100% Pork</td>
<td>1% beef</td>
</tr>
<tr>
<td>Beef lean (g)</td>
<td>-</td>
<td>12</td>
</tr>
<tr>
<td>Beef fat (g)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pork lean (g)</td>
<td>700</td>
<td>688</td>
</tr>
<tr>
<td>Pork fat (g)</td>
<td>200</td>
<td>200</td>
</tr>
<tr>
<td>ice/water (g)</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>milk powder (g)</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>salt</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>seasonings</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
3.7.3 Effect of milk powder on the detection of beef in pork products

The effect of milk powder, frequently used as a binder, on the detection of beef in pork products was examined by coating a microtitre plate with milk powder diluted in the coating buffer. Five, ten and fifteen percent solutions of milk powder was tested in EIA (section 3.5.9).

3.7.4 Enzyme Immunoassay for the detection of beef and pork in beef and pork products

A similar procedure to that used for identifying PTMA (section 3.5.9) was adopted, except that the antigen preparations were first screened for either beef or pork using appropriate dilutions of goat antisera to TMA of cattle and pig. Initially an antigen dilution of 1/400 was used. Those products that were negative for either beef or pork at 1/400 were tested starting with an antigen dilution of 1/2 and diluted serially to 1/400 in the coating buffer.

For the differentiation of known beef and pork, the reaction was considered positive when the absorbance exceeded 3 times that of the mean of the negative sample (Savingy and Voller, 1980). Pure beef and pork sausage extracts were included in each test as controls.

3.8 Analysis of Enzyme Immunoassay Data

The data of absorbance obtained from the differentiation tests for PTMA were analysed for statistical significance using a paired Student's t-test (Snedecor and Cochran, 1971).
4. RESULTS AND DISCUSSION
4.1 IMMUNODIFFUSION TESTS

4.1.1 Species identification of TMA

Results of species identification of autoclaved meats (TMA) using unabsorbed goat and rabbit antisera to TMA are tabulated in Tables 3 and 4. These results showed that goat and rabbit antisera to TMA gave a wide range of cross-reactions with various TMA. Rabbit antisera to TMA showed a wider range of cross-reactions than goat antisera to TMA. This is exemplified by comparing rabbit antiserum to TMA of kongoni which cross-reacted with all TMA of the bovidae, while goat antiserum to the same TMA cross-reacted only with TMA of topi, oryx, wildebeest, Thomson's gazelle and sheep (Tables 3 and 4). Only goat antisera to TMA of camel, dog, horse, impala, warthog and wildebeest were species-specific without absorptions (Table 4).

In an experiment designed to check whether antigens similar to or identical with TMA were present in serum, it was found that unabsorbed goat and rabbit antisera to TMA of kongoni reacted with kongoni serum, giving a reaction of identity with the inner precipitin line of kongoni TMA (Fig. 4). This shows that kongoni serum shares some antigenic determinants with kongoni TMA. Using unabsorbed rabbit antiserum to TMA of kongoni, it was observed that TMA of goat, Grant's gazelle and Thomson's gazelle also reacted with the same antiserum giving a reaction of identity with the same precipitin line given by kongoni serum. This
Table 3: Results of immunodiffusion tests using unabsorbed rabbit antisera to TMA and TMA of other species

<table>
<thead>
<tr>
<th>TMA of</th>
<th>Buffalo</th>
<th>Cattle</th>
<th>Bushbuck</th>
<th>Eland</th>
<th>Goat</th>
<th>T. gazelle</th>
<th>Impala</th>
<th>Kongoni</th>
<th>Topi</th>
<th>Wildebeest</th>
<th>Oryx</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>*</td>
<td>*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Cattle</td>
<td>*</td>
<td>*</td>
<td>-</td>
<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bushbuck</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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</tr>
<tr>
<td>Eland</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Goat</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sheep</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Impala</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>T. gazelle</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
<td>-</td>
</tr>
<tr>
<td>G. gazelle</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kongoni</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Topi</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
<td>Wildebeest</td>
<td>+</td>
<td>-</td>
<td>*</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Oryx</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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Key:  +  = reaction of partial identity  
      -  = no reaction  
      *  = reaction of identity  

G. gazelle = Grant's gazelle  
T. gazelle = Thomson's gazelle  
TMA = thermostable muscle antigen
Table 4: Results of immunodiffusion tests using unabsorbed goat antisera to TMA and TMA of various species

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Key: + = reaction of partial identity  
- = no reaction  
* = reaction of identity  
TMA = thermostable muscle antigen
Fig. 4: Results of immunodiffusion tests using unabsorbed goat and rabbit antisera to TMA of kongoni

(a) = Rabbit antiserum to
TMA of kongoni
1 = Kongoni TMA
2 = Kongoni serum
3 = Thomson's gazelle TMA
4 = Kongoni
5 = Grant's gazelle
6 = Goat

(a) = Goat antiserum to
TMA of kongoni
1 = Kongoni TMA
2 = Kongoni serum
3 = Thomson's gazelle TMA
4 = Kongoni
5 = Grant's gazelle
6 = Goat
means that the epitopes detectable in kongoni serum by the
two antisera to TMA of kongoni are similar, if not identical
to those that are shared between the TMA of goat, Grant's
gazelle, Thomson's gazelle and kongoni.

Since the antisera to the TMA of kongoni raised in goat
and rabbit recognized antigenic components of kongoni serum
as similar to or identical with those of the TMA of goat,
Grant's gazelle and Thomson's gazelle, absorption of the
antisera to TMA with homologous serum abolished
cross-reactions with TMA of unrelated species.

Milgrom et al. (1963) described the presence of a
thermostable adrenal gland antigen that was detectable in
homologous and heterologous adrenal gland preparations using
rabbit antisera to thermostable adrenal gland preparations of
man, pig and horse. The 'TMA-like' antigen demonstrated in
kongoni serum and showing reactions of identity with the TMA
of heterologous and homologous species, may be compared to
the adrenal antigens described by Milgrom et al. (1963) with
regard to cross-reactivity.

The results of immunodiffusion tests using absorbed goat
and rabbit antisera to TMA of various species are shown in
Tables 5 and 6. Absorption of the goat antisera to TMA with
copolymerized serum proteins, rendered most of the goat
antisera to TMA specific, with the exception of goat antisera
to TMA of kongoni, topi, Thomson's gazelle and Grant's
**Table 5:** Results of immunodiffusion tests using rabbit antisera to TMA absorbed with copolymerized serum proteins and tested with TMA of various species

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**Key:** * = reaction of identity  
+ = reaction of partial identity  
- = no reaction  
TMA = thermostable muscle antigen  
G. gazelle = Grant's gazelle  
T. gazelle = Thomson's gazelle
Table 6: Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with TMA of various species

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Key: * = reaction of identity
      - = no reaction

TMA = thermostable muscle antigen
gazelle (Table 6). The majority of the rabbit antisera to TMA retained a wide range of cross-reactions even after extensive absorptions (Table 5). Only rabbit antisera to the TMA of oryx and camel were rendered species-specific using the same absorption procedure. Goat antisera to TMA of cattle, buffalo and bushpig demonstrated species specificity when tested with TMA of closely related species (Fig. 5 and 6, 7 and 8, 9 and 10).

Doberstein and Greuel (1982) using rabbit antisera to serum of eland, springbok and impala failed to differentiate between the meat of the closely related eland and kudu in immunodiffusion tests. Kamiyama et al. (1978b) were unable to differentiate between the meat of sheep and goat using rabbit antiserum to goat albumin in passive haemaglutination tests. Similarly, Kang’ethe et al. (1982) using rabbit antiserum to sheep albumin failed to differentiate between the meat of sheep and goat in an enzyme-linked immunosorbent assay.

These results show that antisera to TMA of bovidae raised in rabbits are less specific while antisera to the same raised in related species show a higher degree of specificity. These results are comparable to those reported by Lindqvist et al. (1982).

Although absorbed rabbit antisera to TMA proved to be less species-specific for the purpose of species identification of
Fig. 5: Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to TMA of cattle

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<th>Cattle</th>
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Fig. 6: Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to TMA of cattle

1 = Cattle TMA
2 = Kongoni "
3 = Topi "
4 = Wildebeest "
5 = Dog "
6 = Horse "

1 = Cattle TMA
2 = Pig "
3 = Warthog "
4 = Camel "
5 = Bushpig "
6 = Buffalo "
Fig. 7: Results of immunodiffusion tests using goat antiserum to TMA of buffalo absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to TMA of buffalo

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1 = Buffalo TMA     1 = Buffalo TMA
2 = Sheep          2 = Bushbuck
3 = Goat           3 = Waterbuck
4 = Cattle         4 = Impala
5 = Eland          5 = Thomson's gazelle
6 = Oryx           6 = Grant's gazelle
Fig. 8: Results of immunodiffusion tests using goat antiserum to TMA of buffalo absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to

TMA of buffalo

1 = Buffalo TMA
2 = Kongoni
3 = Topi
4 = Wildebeest
5 = Dog
6 = Horse

1 = Buffalo TMA
2 = Pig
3 = Warthog
4 = Bushpig
5 = Camel
6 = Cattle
Fig. 9: Results of immunodiffusion test using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to

TMA of bushpig

1 = Bushpig TMA  
2 = Sheep  
3 = Goat  
4 = Buffalo  
5 = Eland  
6 = Oryx

1 = Bushpig  
2 = Waterbuck  
3 = Bushbuck  
4 = Impala  
5 = Thomson's gazelle  
6 = Grant's gazelle
Fig. 10: Results of immunodiffusion test using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with TMA of various species

(a) = Goat antiserum to

TMA of bushpig

1 = Bushpig TMA
2 = Kongoni "
3 = Topi "
4 = Wildebeest "
5 = Dog "
6 = Horse "

1 = Bushpig TMA
2 = Pig "
3 = Warthog "
4 = Camel "
5 = Cattle "
6 = Cattle "
meat, the rabbit antisera could be used to differentiate reliably meats of distantly related species (Table 5). In addition, the antisera to TMA of topi, Grant's gazelle and warthog could be used to check initially for the presence of bovidae and non-bovidae meats in samples presented for analysis (Table 7).

4.1.2 Species Identification of CMA

Most of the absorbed goat antisera to TMA were shown to be species-specific in their reactions with CMA, except goat antisera to TMA of cattle, kongoni, bushpig, pig, topi, Thomson's gazelle and Grant's gazelle. Absorbed goat antiserum to TMA of cattle reacted with buffalo CMA, while goat antiserum to TMA of buffalo did not react with TMA or CMA of cattle (Tables 6 and 8). Similar results were observed with goat antiserum to TMA of pig and the TMA and CMA of bushpig and warthog (Tables 6 and 8).

Reactions observed with goat antisera to TMA of topi, bushpig and horse with CMA of other species are shown in Fig. 11 and 12, 13 and 14, 15 and 16. These results show that absorbed goat antiserum to TMA of horse was species-specific, but antisera to TMA of topi and bushpig cross-reacted with CMA of related species.

Absorbed goat antisera to TMA of kongoni, topi, Thomson's gazelle and Grant's gazelle reacted with TMA and CMA of other
Table 7: Results of immunodiffusion tests using absorbed rabbit antisera to TMA, which could be used to distinguish meat from bovidae and non-bovidae

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Key: * = reaction of identity
+ = reaction of partial identity
- = no reaction
TMA = thermostable muscle antigen
Table 8: Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with CMA of various species

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Key: + = reaction of identity
     - = no reaction

CMA = cooked meat antigen
TMA = thermostable muscle antigens
Fig. 11: Results of immunodiffusion tests using goat antiserum to TMA of topi absorbed with copolymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to TMA of topi

1 = Topi CMA
2 = Sheep "
3 = Goat "
4 = Kongoni "
5 = Cattle "
6 = Oryx "

1 = Topi CMA
2 = Eland "
3 = Wildebeest "
4 = Waterbuck "
5 = Bushbuck "
6 = Kongoni "
Fig. 12: Results of immunodiffusion tests using goat antiserum to TMA of topi absorbed with polymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to TMA of topi

1 = Topi                   CMA                      1 = Topi                   CMA
2 = Thomons's gazelle      "                         2 = Warthog      "
3 = Grant's gazelle        "                         3 = Buffalo      "
4 = Dog                    "                         4 = Bushpig      "
5 = Pig                    "                         5 = Camel       "
6 = Horse                  "                         6 = Kongoni     "
Fig. 13: Results of immunodiffusion tests using goat antiserum to TMA of warthog absorbed with copolymerized serum proteins and tested with CMA of various species.

(a) = Goat antiserum to TMA of warthog.

1 = Warthog CMA
2 = Sheep
3 = Goat
4 = Kongoni
5 = Cattle
6 = Oryx

1 = Warthog CMA
2 = Eland
3 = Wildebeest
4 = Waterbuck
5 = Bushbuck
6 = Impala
Fig. 14: Results of immunodiffusion tests using goat antiserum to TMA of warthog absorbed with copolymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to TMA of warthog

1 = Warthog
2 = Thomson's gazelle
3 = Grant's gazelle
4 = Dog
5 = Camel
6 = Dog

CMA

1 = Warthog
2 = Pig
3 = Buffalo
4 = Topi
5 = Kongoni
6 = Bushpig
Fig. 15: Results of immunodiffusion tests using goat antiserum to TMA of horse absorbed with copolymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to TMA of horse

1 = Horse CMA
2 = Sheep "
3 = Goat "
4 = Kongoni "
5 = Cattle "
6 = Oryx "
1 = Horse CMA
2 = Eland "
3 = Wildebeest "
4 = Waterbuck "
5 = Bushbuck "
6 = Impala "
Fig. 16: Results of immunodiffusion tests using goat antiserum to TMA of horse absorbed with copolymerized serum proteins and tested with CMA of various species

(a) = Goat antiserum to

TMA of horse

1 = Horse CMA  
2 = Thomson's gazelle "  
3 = Grant's gazelle "  
4 = Warthog "  
5 = Camel "  
6 = Dog "

1 = Horse CMA  
2 = Pig "  
3 = Buffalo "  
4 = Bushpig "  
5 = Topi "  
6 = Kongoni "
species (Table 6 and 8), while goat antisera to TMA of cattle, pig and bushpig reacted only with CMA and not with TMA of closely related species (Table 6 and 8).

These results indicate that the species-specificity observed when goat antisera to TMA is tested with TMA of various species may possibly be a result of emergence of novel antigens due to the heat treatment. Hirose and Osler (1967), Henney and Ishizaka (1968) and Karpas et al. (1970) reported the emergence of new antigens arising during the heating process. However, if this were true, one would have expected a wider species cross-reactivity between goat antisera to TMA of bovidae and CMA because the TMA precursors present in fresh meat of these species could be similar if not identical. It is possible that the autoclaving step may produce conformational changes which expose the species-specific epitopes masked by non-specific determinants common in CMA of closely related species.

4.1.3 Species identification of FMA

The results given in Table 9, show that absorbed goat antisera to most of the TMA were species-specific, except antisera to TMA of cattle, bushpig, kongoni, warthog, Grant's gazelle and Thomson's gazelle which reacted with FMA of closely related species.

Absorbed goat antisera to TMA of Thomson's gazelle cross-reacted with FMA of Grant's gazelle but not with the
Table 9: Results of immunodiffusion tests using goat antisera to TMA absorbed with copolymerized serum proteins and tested with FMA of various species

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<th>FMA of</th>
<th>Buffalo</th>
<th>Cattle</th>
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<th>Sheep</th>
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Key: + = reaction of identity  FMA = fresh meat antigen
- = no reaction  TMA = thermostable muscle antigens
FMA of goat, sheep and impala (Fig. 17 and 18) which are closely related. Absorbed goat antisera to TMA of bushpig cross-reacted with FMA of pig and warthog but not with FMA of other species (Fig. 19 and 20).

Reactions of goat antisera to TMA with FMA revealing a high degree of species-specificity strongly suggests that TMA are naturally occurring antigens of muscle tissue and are not new antigens arising from heat denaturation.

4.1.4 Species identification of organs

Results of immunodiffusion tests using absorbed goat antisera to TMA of cattle, pig, kongoni and Thomson's gazelle with TOA of liver, heart and kidney are shown in Table 10. These results show that antisera to TMA can also be used to identify the species of origin of organs.

Results of immunodiffusion tests using absorbed goat antiserum to TMA of cattle and TOA of cattle liver, heart and kidney are shown in Fig. 21. These results show that species-specific TMA of cattle exist as three distinct antigens. One antigen that is species-specific, and found in the striated muscles (skeletal and cardiac) and in other organs (kidney and liver). The second antigen was found to be restricted only to the striated muscles (skeletal and cardiac) while a third antigen was found to be specific for skeletal muscle. Similar results were obtained when goat antisera to TMA of kongoni, pig and Thomson's gazelle reacted with their respective TOA.
Fig. 17: Results of immunodiffusion tests using goat antiserum to TMA of Thomson's gazelle absorbed with copolymerized serum proteins and tested with FMA of various species.

(a) = Goat antiserum to

<table>
<thead>
<tr>
<th>TMA of Thomson's gazelle</th>
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<tbody>
<tr>
<td>1 = Thomson's gazelle FMA</td>
</tr>
<tr>
<td>2 = Sheep</td>
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<td>3 = Goat</td>
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<td>4 = Cattle</td>
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<tr>
<td>5 = Eland</td>
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<td>6 = Oryx</td>
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</table>

<table>
<thead>
<tr>
<th>1 = Thomson's gazelle FMA</th>
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<tbody>
<tr>
<td>2 = Waterbuck</td>
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<td>3 = Bushbuck</td>
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<td>4 = Impala</td>
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<tr>
<td>5 = Grant's gazelle</td>
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<tr>
<td>6 = Buffalo</td>
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</table>
Fig. 18: Results of immunodiffusion tests using goat antiserum to TMA of Thomson's gazelle absorbed with copolymerized serum proteins and tested with FMA of various species

(a) = Goat antiserum to TMA of Thomson's gazelle

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<tr>
<th></th>
<th>Thomson's gazelle FMA</th>
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<th>Thomson's gazelle FMA</th>
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<td>1 = Thomson's gazelle FMA</td>
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Fig. 19: Results of immunodiffusion tests using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with FMA of various species

(a) = Goat antiserum to TMA of bushpig

1 = Bushpig FMA
2 = Sheep "
3 = Goat "
4 = Cattle "
5 = Eland "
6 = Oryx "
1 = Bushpig FMA
2 = Waterbuck "
3 = Bushbuck "
4 = Impala "
5 = Grant's gazelle "
6 = Thomson's gazelle "
Fig. 20: Results of immunodiffusion tests using goat antiserum to TMA of bushpig absorbed with copolymerized serum proteins and tested with FMA of various species.

(a) = Goat antiserum to TMA of bushpig

1 = Bushpig FMA
2 = Kongoni "
3 = Topi "
4 = Wildebeest "
5 = Dog "
6 = Pig "
1 = Bushpig FMA
2 = Warthog "
3 = Horse "
4 = Camel "
5 = Buffalo "
6 = Cattle "

Table 10: Species identification of internal organs using goat antisera to TMA absorbed with copolymerized serum proteins and tested with TOA in immunodiffusion tests

<table>
<thead>
<tr>
<th>Organ</th>
<th>TOA of</th>
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<th>Kongoni</th>
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<td>Pig</td>
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</table>

Key:
* = reaction of identity
- = no reaction
TOA = thermostable organ antigens
TMA = thermostable muscle antigens
Fig. 21: Results of immunodiffusion tests using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins and tested with TOA of cattle

(a) = Goat antiserum to TMA of cattle

1 = cattle TMA

2 = cattle liver TOA

3 = cattle kidney

4 = cattle TMA

5 = cattle heart TOA

6 = cattle lung
Milgrom et al. (1963) described the presence of two adrenal thermostable antigens, which were species and adrenal gland specific. In this study, species-specific TMA has been shown to consist of at least three antigens. The adrenal gland specific thermostable antigen may be compared to the skeletal muscle specific antigen.

4.2 **ENZYME IMMUNOASSAY (EIA)**

4.2.1 **Purification of TMA**

The fractions from the Sephadex G-200 and G-75 columns that contained the PTMA as detected by EIA, gave low absorbances at 280 nm. The fractions with high absorbances at 280nm were shown to contain no specific activity to goat antiserum to TMA of buffalo (Figs. 22 and 23).

The proteins represented by the high absorbances at 280 nm in the fractions from both Sephadex G-75 and G-200 columns and shown not to be antigenic, would compete for binding to the microtitre plate with the PTMA and result in inconsistent and irregular adsorptions observed using TMA extracted according to the method of Milgrom and Witebsky (1962). While these TMA may provide antigens suitable for use in immunodiffusion tests, such antigens are unsuitable for use in EIA. Although ultrafiltration alone using PM30 is capable of some degree of purification, it is not recommended as the only method for the preparation of PTMA for use in EIA.
Fig. 22: Fractionation of buffalo TMA on Sephadex G-200 column

Three ml of buffalo TMA (11.50 mg protein/ml) were length fractionated on Sephadex G-200 column, 94.4 cm and 2.6 cm. diameter. The optical density of fractions was read at 280 nm ( — — — ) and their antigen contents determined by a standard EIA (OD 410 nm — — — — — — ) .
Three ml of buffalo TMA (11.50 mg protein/ml) were fractionated on Sephadex G-75 column, length 35.5 cm and 2.6 cm diameter. The optical density of the fractions was read at 280 nm (---o---o--) and their antigen contents determined by a standard EIA (OD 410 nm ——o—)
4.2.2 Effect of coating buffer on the adsorption of PTMA to microtitre plates

Of the three coating buffers, the carbonate-bicarbonate buffer (0.05M, pH 9.6) resulted in more efficient adsorption of the antigen (buffalo PTMA) to the microtitre plate than the other two. Use of carbonate-bicarbonate buffer resulted in forty times more antigen being adsorbed to the microtitre plate than either 1/100 PBS + 2% PEG or 1/100 PBS + 2% PEG + 0.5 M NaCl (Fig. 24).

4.2.3 Optimal dilutions of PTMA, antiserum and conjugate for use in enzyme immunoassay

Table 11 shows the results of checker-board titrations for determining optimal dilutions of PTMA, antiserum and rabbit anti-goat IgG-glucose oxidase conjugate for use in EIA.

4.2.4 Effect of gelatin on the adsorption of the antigen to microtitre plates

Thermostable muscle antigens when used to coat microtitre plates resulted in inconsistent and irregular adsorption to the plates. These antigens were also observed to gel at 4°C, indicating that they contained high amounts of gelatin. This gelatin was thought to be the cause of poor adsorption of the PTMA on the plates.

Qualitative assay of hydroxyproline in the TMA extracted according to the method of Milgrom and Witebsky (1962), PTMA,
Fig. 24: **Effect of three buffers on the adsorption of**

the PTMA to microtitre plates

Buffalo PTMA (100 µg protein/ml) was diluted serially in 0.05 M carbonate-bicarbonate buffer, pH 9.6 (---O---), 1/100 PBS + 2% PEG + 0.5 M NaCl (---O---) and 1/100 PBS + 2% PEG (---*---*---). The dilutions were applied to microtitre plates and the adsorption of antigen determined by a standard EIA.
<table>
<thead>
<tr>
<th>PTMA</th>
<th>PTMA for optimal coating (µg protein/ml)</th>
<th>Absorbed goat antiserum dilution</th>
<th>Dilution of the rabbit anti-goat IgG glucose oxidase conjugate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffalo</td>
<td>100</td>
<td>Goat 340; 1:40</td>
<td>1:1000</td>
</tr>
<tr>
<td>Cattle</td>
<td>100</td>
<td>Goat 343; 1:10</td>
<td>1:3000</td>
</tr>
<tr>
<td>Bushbuck</td>
<td>100</td>
<td>Goat 831; 1:10</td>
<td>1:1600</td>
</tr>
<tr>
<td>Eland</td>
<td>100</td>
<td>Goat 346; 1:40</td>
<td>1:1600</td>
</tr>
<tr>
<td>Grant's Gazelle</td>
<td>100</td>
<td>Goat 344; 1:10</td>
<td>1:400</td>
</tr>
<tr>
<td>Thomson's gazelle</td>
<td>100</td>
<td>Goat 347; 1:10</td>
<td>1:400</td>
</tr>
<tr>
<td>Impala</td>
<td>100</td>
<td>Goat 342; 1:10</td>
<td>1:800</td>
</tr>
<tr>
<td>Kongoni</td>
<td>12.5</td>
<td>Goat 826; 1:100</td>
<td>1:1500</td>
</tr>
<tr>
<td>Topi</td>
<td>100</td>
<td>Goat 829; 1:80</td>
<td>1:1500</td>
</tr>
<tr>
<td>Wildebeest</td>
<td>100</td>
<td>Goat 827; 1:10</td>
<td>1:3000</td>
</tr>
<tr>
<td>Waterbuck</td>
<td>12.5</td>
<td>Goat 832; 1:20</td>
<td>1:1500</td>
</tr>
<tr>
<td>Oryx</td>
<td>100</td>
<td>Goat 856; 1:40</td>
<td>1:800</td>
</tr>
<tr>
<td>Pig</td>
<td>6.25</td>
<td>Goat 863; 1:640</td>
<td>1:3000</td>
</tr>
<tr>
<td>Warthog</td>
<td>100</td>
<td>Goat 869; 1:80</td>
<td>1:1500</td>
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<tr>
<td>Horse</td>
<td>12.5</td>
<td>Goat 897; 1:40</td>
<td>1:3000</td>
</tr>
<tr>
<td>Camel</td>
<td>100</td>
<td>Goat 837; 1:40</td>
<td>1:3000</td>
</tr>
</tbody>
</table>
fractions of Sephadex G-200 that showed no antigenic activity in EIA, and the concentrated extracts of the modified procedure before chromatography, showed that TMA, concentrated extracts before chromatography and the Sephadex G-200 fractions that contained no antigen contained hydroxyproline while the PTMA had no hydroxyproline. This was an indication of the presence of gelatin in these preparations.

Machlik and Draudt (1963) showed that when collagen is heated to about 60°C it shortens and is converted into a more soluble form. The percentage of collagen solubilized by heating has been shown to increase gradually as the temperature is increased from 60°C to 90°C (Snowden and Weidemann, 1978). Gelatin formation has been shown to be swift with pressure cooking (Bendall, 1946). The TMA extraction procedure of Milgrom and Witebsky (1962) provides favourable conditions for gelatin formation as it includes cooking and autoclaving steps.

Increasing the amount of gelatin in the buffalo PTMA, resulted in an increased inhibitory effect on the adsorption of the PTMA to the microtitre plate. A gelatin concentration of 5 mg/ml resulted in 100% inhibition of the antigen adsorption to the plate (Fig. 25).

The presence of gelatin in TMA extracts and Sephadex G-200 fractions as demonstrated by the assay for hydroxyproline and the contaminating proteins present in TMA would inhibit the adsorption of the PTMA to the plates.
Fig. 25: The inhibitory effect of gelatin on the adsorption of PTMA to microtitre plates

A fixed amount of buffalo PTMA (42.7 µg protein/ml) was mixed with increasing amounts of commercial gelatin and applied to microtitre plates. A standard EIA was performed and the percent inhibition calculated.
Where indirect EIA is to be used, purification of the TMA to yield PTMA is necessary in order to remove these contaminating proteins and prevent irregular adsorptions.

4.2.5 **Differentiation of PTMA using enzyme immunoassay**

Several of the absorbed goat antisera to TMA showed a wide range of cross-reactions with PTMA of several species in quantitative EIA. However, goat antisera to TMA of topi, buffalo, camel, horse and pig were shown to be species-specific (Table 12). These goat antisera to TMA were able to distinguish between heterologous and homologous PTMA at \( p < 0.05 \). Nine other antisera showed various degrees of cross-reactions with 2 to 10 heterologous PTMA. These reactions with heterologous PTMA were nearly equal to those given by the homologous PTMA. Absorption of the antisera with the highly cross-reacting PTMA resulted in abolition of the homologous reaction.

Goat antiserum to TMA of cattle and eland cross-reacted with 13 and 15 heterologous PTMA respectively. Reactions with these PTMA did not allow unequivocal differentiation of the homologous from the heterologous PTMA. However, goat antiserum to TMA of cattle gave weak reactions with PTMA of topi, pig, horse and Thomson's gazelle which enabled differentiation between homologous and these heterologous PTMA at \( p \leq 0.05 \). Similarly, goat antiserum to TMA of eland
Table 12: Species differentiation of PTMA in enzyme immunoassay

<table>
<thead>
<tr>
<th>PTMA of</th>
<th>Buffalo</th>
<th>Cattle</th>
<th>Bushbuck</th>
<th>Eland</th>
<th>Grant's Gazelle</th>
<th>Impala</th>
<th>Kongoni</th>
<th>Topi</th>
<th>Wildebeest</th>
<th>Waterbuck</th>
<th>Oryx</th>
<th>Pig</th>
<th>Warthog</th>
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<tr>
<td>Buffalo</td>
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Key: Significance level  | DMA = thermostable muscle antigen  | PTMA = partially purified thermostable muscle antigen  | NS = not significant  | A = homologous system
--- | --- | --- | --- | ---
*** = P<0.001  |  |  |  |  
** = P<0.01   |  |  |  |  
* = P<0.05    |  |  |  |  
--- | --- | --- | --- | ---
reacted with PTMA of cattle and horse giving reactions that
allowed differentiation between the PTMA of eland from those
of cattle and horse at $p < 0.05$.

While goat antisera to TMA of eland and cattle reacted
with most of PTMA of other species giving reactions that did
not allow differentiation of homologous from heterologous
PTMA, the PTMA of cattle and eland reacted with most of the
goat antisera to TMA of other species, giving reactions that
allowed these PTMA to be differentiated from the PTMA of
other species (Table 12).

In instances where a single antiserum failed to
distinguish between homologous and heterologous PTMA, species
identification could be achieved in EIA by employing a number
of goat antiserum to TMA of several species. Using this
technique, goat antisera to TMA provided reagents that were
capable of correctly identifying the PTMA of all the eighteen
species examined.

Because of their exquisite specificity, monoclonal
antibodies to TMA would be more suitable for species
identification than the polyclonal antisera used in this
study. Monoclonal antibodies to heated ($70^\circ C$) meat
extracts of cattle and kangaroo have been used successfully
to identify the species of origin of fresh and heated meat in
EIA by Georlich and Greuel (1986). However, it is
conceivable that immunization of additional animals and
animals of different species especially within the bovidae would yield polyclonal antisera with sufficient specificity for use in EIA. Such polyclonal antisera has been raised to whole serum of vertebrate species by Rurangirwa et al. (1986).

In an experiment to determine the lowest detectable level of buffalo PTMA in beef, impala in goat and warthog in pig, the EIA failed to detect 1% and 10% buffalo in cattle, impala in goat and warthog in pig at $p < 0.05$. Lower level of adulteration 0.5% has been detected in fresh meats using sandwich EIA by Jones and Patterson (1985). These authors used fresh meats from remotely related animal species, cattle and pig. In this study, heat-treated meats (PTMA) of unrelated species were easily differentiated (Table 12). If such species were choosen, lower levels of admixtures could have been detected.

4.2.6 Determination of the molecular weight of the PTMA using gel filtration

The buffalo PTMA was eluted just after the blue dextran representing the void volume of the Sephadex G-200 column (Fig. 26). Using the molecular weight standard curve (Fig. 27), the molecular weight of the PTMA was calculated to be 210 kd.
Fig. 26: Elution profile from Sephadex G-200 of buffalo PTMA and molecular weight markers

Three ml of buffalo TMA (11.5 mg protein/ml) was fractionated on a Sephadex G-200 (94.4 cm long and 2.6 cm diameter) which had been calibrated with a mixture of blue dextran\(^{(1)}\) (1 mg, approx. 2,000,000 D); human IgG\(^{(2)}\) (20 mg, approx. 150,000 D) and bovine serum albumin\(^{(3)}\) (40 mg, approx. 67,000 D) on the basis of OD 280 nm (— — — — ). PTMA was detected in the fractions by a standard EIA (OD 410 nm — — — — ).
The Kav of the molecular weight markers (1 = human IgG, 150,000 D, Kav = 0.15 and 2 = bovine serum albumin, 67,000 D, Kav = 0.36) were plotted against their corresponding molecular weight on a semilogarithm graph paper. The molecular weight of buffalo PTMA (----, Kav = 0.06) was read off the curve.
4.3 USE OF ENZYME IMMUNOASSAY FOR SPECIES IDENTIFICATION OF MEAT IN MEAT PRODUCTS

4.3.1 Detection of beef and pork in pork and beef products

Of the 46 samples labelled pork, 23 (50%) were positive for beef (Table 13). Included in these 23 samples were 8 samples that gave negative reactions for pork.

Twenty three (52.3%) of the 44 samples labelled beef gave positive reactions for pork (Table 13). Among these were 6 samples that were negative for beef. Three of these gave negative reactions for both beef and pork. These products gave weak positive reactions for both beef and pork.

The mixing of pork with beef was detected in 50% of the pork products from both factories. Mixing of beef with pork was found in 22/28 (78.6%) of the beef products from factory A, and only in 1/16 (6.3%) of the beef products from factory B (Table 14).

This high rate of addition of pork to beef and vice-versa in products from both factories cannot be referred to as adulteration, since in this country specifications for the manufacture of beef and pork sausages allows inclusion of other unspecified meats. For beef products, the allowed minimum lean beef should at least be 32.5% while pork products should contain at least 52% lean pork (Kenya Bureau of Standards, 1978).

In an experiment to determine the lowest detectable level of beef in pork sausages, the EIA detected 5% beef in pork at
Table 13: Results of ELA for the detection of beef and pork meat in commercial pork and beef products

<table>
<thead>
<tr>
<th>Product</th>
<th>Number</th>
<th>No. positive for pork (%)</th>
<th>No. positive for beef (%)</th>
<th>No. positive for beef and pork (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Beef</td>
<td>44</td>
<td>23 (52.3)</td>
<td>38 (86.4)</td>
<td>23 (52.3)</td>
</tr>
<tr>
<td>Pork</td>
<td>46</td>
<td>38 (82.6)</td>
<td>23 (50)</td>
<td>23 (50)</td>
</tr>
</tbody>
</table>
Table 14: Results of ElA showing detection of beef and pork in meat products from two factories

<table>
<thead>
<tr>
<th>Factory</th>
<th>Product label</th>
<th>Number of products examined</th>
<th>Tested for</th>
<th>Number positive (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>beef</td>
<td>28</td>
<td>Pork</td>
<td>22 (78.6)</td>
</tr>
<tr>
<td></td>
<td>pork</td>
<td>24</td>
<td>Beef</td>
<td>12 (50)</td>
</tr>
<tr>
<td>B</td>
<td>Beef</td>
<td>16</td>
<td>Pork</td>
<td>1 (6.3)</td>
</tr>
<tr>
<td></td>
<td>Pork</td>
<td>22</td>
<td>Beef</td>
<td>11 (50)</td>
</tr>
</tbody>
</table>
P < 0.01 but not 1% beef in pork even at P < 0.05 (Fig. 28). Kang'ethe et al. (1982) detected 3% horse meat in beef, while Patterson and Spencer (1985), and Jones and Patterson (1985) reported that they could detect 0.1% kangaroo meat in beef and 0.5% horse meat in beef using a sandwich EIA. The methods described earlier by Kang'ethe et al. (1982), Patterson and Spencer (1985) and Jones and Patterson (1985) were designed to identify the species of origin of unheated meats and are therefore unsuitable for heat-treated products. Use of antisera to TMA allows application of the EIA to be used for the examination of raw, unheated products and also for commercially heat-treated products.

4.3.2. **Effect of milk powder on the detection of beef in pork products**

Milk powder 5% (same level as the one incorporated in the sausage) when diluted in the coating buffer did not result in false positive reactions for beef. Increasing the quantity of milk powder to 15% in the coating buffer did not produce significant differences in the absorbance at 410 nm when compared to those obtained with 5% milk powder.

Di Antonio et al. (1984) were unable to differentiate between pork sausages containing bovine milk powder with those that contained bovine meat in immunodiffusion tests using anti-bovine serum obtained from a commercial firm. The
Fig. 28: Detection by EIA of beef in experimentally prepared pork sausages using goat antiserum to TMA of cattle absorbed with copolymerized serum proteins.

Dilutions of phosphate buffered saline extracts of pure beef sausage (---○---○--) pure pork sausage (---△---△--), 10% beef in pork sausage (---□---□--), 5% beef in pork sausage (---×---×--), and 1% beef in pork sausage (---*---*--) were tested for the presence of beef using a standard EIA.
discrepancy between these results and those of Di Antonio et al. (1984) may be due to differences in the specificities of the two antisera as well as the methodologies. Hofmann (1974) showed that electrophoretic patterns of meat, soya bean protein, milk powder and egg white in SDS-PAGE were so different that the presence of these non-meat proteins in meat products did not impair detection of meat proteins. The observation that milk powder in pork sausages did not impair the detection of beef in pork sausages means that goat antisera to TMA can be used to detect adulteration of meat products containing such additives without the risk of false positive reactions.
5. CONCLUSIONS
Identification of the species of origin of fresh, cooked and autoclaved meats is a challenging and a complex problem for the food analyst and forensic scientist. While species identification of fresh meat has been achieved using a variety of techniques, identification of the species of origin of heat-treated meat and meat products has remained a persistent problem in the food industry. The objectives of this study were to demonstrate the existence of TMA and use the same to identify the species of origin of autoclaved, cooked and fresh meat and meat products using serological techniques.

The following conclusions can be drawn based on the results obtained in this study.

1. By selection of zoologically related animals for the production of antisera to TMA, it is possible to produce antisera with adequate specificity for the purpose of identification of the species of origin of cooked and fresh meat as well as of organs.

2. Antisera to TMA gave reactions of identity with homologous TMA, CMA, FMA and TOA demonstrating that TMA are not novel antigens but are present in both heat-treated and fresh meats and also in organs.

3. Thermostable muscle antigens exist as three clearly distinct components.
(a) a species-specific antigen, found in striated muscle and organs of homologous species.

(b) species and striated muscle specific antigen, found in skeletal and cardiac muscles.

(c) species and tissue specific antigen, found only in skeletal muscle.

4. Although rabbit antiserum to TMA lacked sufficient specificity, antiserum to TMA of topi, warthog and Grant's gazelle could be used to distinguish between meats from bovidae or non-bovidae.

5. Gelatin and other contaminating proteins found in the TMA extracted according to the method of Milgrom and Witebsky (1962) are undesirable in EIA as they inhibit adsorption of the PTMA to microtitre plates. A modified procedure for the extraction of PTMA yielded antigens suitable for use in EIA.

6. Differentiation of PTMA by EIA could be achieved using a selection of antiserum produced against TMA of several species.

7. Antiserum to TMA have been shown to be specific for meat proteins. They can, therefore, be used to identify the species of origin of meat in meat products which may contain non-meat proteins in EIA without the risk of false positive reactions.

8. Antiserum to TMA are and will be indispensable in species identification of heat-treated as well as fresh meats.
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APPENDIX
1

List of buffers and solutions used for immunodiffusion tests

1.1 0.15M Phosphate buffer, pH 7.4

Di-sodium hydrogen phosphate (anhydrous) 21.3 g
Di-sodium dihydrogen phosphate 23.4 g
Distilled water upto 1000 ml

1.2 Trisodium citrate buffer for washing slides

Trisodium citrate 3 g
Sodium azide 1 g
Distilled water made upto 1000 ml

1.3 1% agar in phosphate buffered saline pH 7.4, for immunodiffusion tests

Purified Oxoid agar 2 g
Sodium azide 0.2 g
Phosphate buffered saline 200 ml
APPENDIX 2

2 Protein staining and destaining solutions

2.1 Coomassie Brilliant Blue 250R solution

Coomassie Brilliant Blue 250R 10 g
Ethanol 900 ml
Glacial acetic acid 200 ml
Distilled water 900 ml

2.2 Ponceau S solution

Ponceau S 2 g
1 M Acetic acid 1000 ml
0.1 M Sodium acetate 1000 ml

2.3 Destaining solution for Coomassie Blue

Ethanol 900 ml
Glacial acetic acid 200 ml
Distilled water 900 ml

2.4 Destaining solution for Ponceau S procedure

3% v/v glacial acetic acid in distilled water
APPENDIX 3

Buffers and solutions used in the preparation of insoluble immunosorbents

3.1 1 mM hydrochloric acid solution for washing the freeze dried cyanogen bromide activated Sepharose 4B

This solution was prepared by diluting 35% concentrated acid Sp. gr. 1.18

3.2 0.1M Sodium bicarbonate solution pH 8.3 with 0.5M sodium chloride (protein coupling buffer)

Sodium hydrogen carbonate 8.40 g
Sodium chloride 29.22 g

8.4 g of sodium hydrogen carbonate were dissolved in 200 mls of distilled water, the pH was adjusted to 8.3 with 1 M NaOH. 29.22 of NaCl was added and stirring continued until the salt had dissolved. The pH was checked again and the volume made to 1000 ml.

3.3 0.2M glycine, pH 8.0 (blocking agent)

Glycine 15.01 g

15.01 g of glycine were dissolved in 200 ml of distilled water and the pH adjusted to 8.0, using 1M HCl. Distilled water was added up to 1000 ml.

3.4 0.1M acetate buffer pH 4.0, containing 0.5M sodium chloride

Sodium acetate 8.2 g
Sodium chloride 29.22 g
Acetic acid 5.77 ml
dissolved in 1000 ml distilled water

3.5 0.1M glycine-HCl buffer pH 2.5 - for regeneration of immunosorbents

Glycine 7.51 g
HCl 0.2 M
7.51 g of glycine were dissolved in 500 ml distilled water and the pH titrated to 2.5 using 0.2 M HCl, then made up to 1000 ml.

3.6 0.1M Tris-HCl buffer, pH 8.5, containing 0.5M NaCl for regenerating immunosorbents

Tris (hydroxymethyl)aminomethane 12.1 g
Sodium chloride 29.22 g

12.1 g Tris and 29.22 g Sodium chloride were dissolved in 500 ml distilled water. The pH was adjusted to 8.5 using 1M HCl, and then made up to 1000 ml.

3.7 0.2M acetate buffer, pH 5.0 - for copolymerizing serum proteins

Sodium acetate 16.41 g
dissolved in 500 ml distilled water

Glacial acetic (17.5 M) 11.42 ml

and the volume made up to 1000 ml
Reagents and solutions used in the Folin-Ciocalteu's method for determination of protein content

4.1 Solution 1
2% sodium carbonate in 0.1N sodium hydroxide.

Solution 2
0.5% copper sulphate in 1% sodium tartrate prepared freshly by mixing equal volumes of double strength reagents.

Solution 3
Alkaline copper solution, prepared by mixing 50 parts of solution 1 with 1 part of solution 2.

4.2 Phenol reagent
Commercial phenol reagent (Fisher Scientific Company, New York) was diluted in the ratio of 5 parts reagent to 4.7 parts of distilled water.

4.3 Bovine serum albumin (BSA)
BSA (No. A-4503, lot no. 107C - 0307 Fraction V, Sigma Chemical Company, St. Louis, USA) was used to prepare the standard curve for the determination of protein content.
Buffers and reagents used for the determination of hydroxyproline

5.1 **L-hydroxyproline** (BDH, Chemicals Ltd., Poole, England). Concentrations ranging from 1-6 ug/ml were prepared in distilled water.

5.2 **Borate buffer pH 8.7 (1M buffer in terms of boric acid)**

- Boric acid: 61.84 g
- Potassium chloride: 225 g

dissolved in 800 ml distilled water.

The pH was adjusted to 8.7 using 1 M KOH and the volume adjusted to 1000 ml.

5.3 **0.112M Alanine solution**

- DL-Alanine (BDH Chemicals Ltd., Poole, England) 10 g

dissolved in 90 ml distilled water, and pH adjusted to 8.7 and the volume adjusted to 1000 ml.

5.4 **Borate-alanine buffer**

100 ml of the borate buffer was mixed with 50 ml alanine solution. The resulting solution was used in 1:10 concentration (0.066 M boric acid).
5.5 0.2M Chloramine-T Solution
Chloramine-T 56.34 g
dissolved in 1000 ml distilled water

5.6 3.6M Sodium thiosulphate solution
Sodium thiosulphate 89.34 g
dissolved in 100 ml distilled water

5.7 Toluene — analytical grade (BDH Chemicals Ltd.,
Poole, England).

5.8 Ehrlich's Reagent
p-dimethylaminobenzaldehyde (BDH Chemicals Ltd.,
Poole, England) 120 g
dissolved in 200 ml absolute ethanol
Concentrated sulphuric acid was added slowly to 200 ml
absolute ethanol in a beaker and the mixture cooled.
The acid ethanol mixture was added to the
p-dimethylaminobenzaldehyde solution in an ice-bath.
APPENDIX 6

Reagent and buffers for enzyme immunoassay

6.1 0.05M carbonate-bicarbonate buffer, pH 9.6 (coating buffer)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium carbonate</td>
<td>1.59 g</td>
</tr>
<tr>
<td>Sodium hydrogen carbonate</td>
<td>2.93 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
</tbody>
</table>

This buffer was prepared fresh every day.

6.2 Buffer used for diluting antiserum

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05M phosphate buffer pH 8.0</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>75 g</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid disodium salt</td>
<td>1 g</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Tween 80</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

The pH was adjusted to 7.5 using 4M NaOH. Normal rabbit serum was added to 5%.

6.3 Buffer used for diluting conjugate

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05M phosphate buffer pH 8.0</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Potassium chloride</td>
<td>75 g</td>
</tr>
<tr>
<td>Ethylenediaminetetraacetic acid disodium salt</td>
<td>1 g</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>2.5 g</td>
</tr>
<tr>
<td>Tween 80</td>
<td>5 ml</td>
</tr>
</tbody>
</table>

The pH was adjusted to 7.5 using 4M NaOH. Normal rabbit serum was added to 5%.
6.4 Substrate buffers

6.4.1 0.05 M ammonium citrate buffer pH 5.0

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citric acid</td>
<td>10.5 g</td>
</tr>
<tr>
<td>Benzoic acid</td>
<td>1 g</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>3 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>approx. 800 ml</td>
</tr>
</tbody>
</table>

The pH was adjusted to 5.0 with concentrated ammonia solution.
The volume was made up to 1000 ml with distilled water.

6.4.2 Substrate solution for glucose oxidase conjugate for one microtitre plate

<table>
<thead>
<tr>
<th>Component</th>
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</tr>
</thead>
<tbody>
<tr>
<td>0.05 M ammonium citrate buffer, pH 5.0</td>
<td>10 ml</td>
</tr>
<tr>
<td>d-Glucose solution (20%)</td>
<td>1 ml</td>
</tr>
<tr>
<td>Horseradish peroxidase type II</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>(1 mg/ml) in ammonium citrate buffer, pH 5.0</td>
<td></td>
</tr>
<tr>
<td>ABTS (2'2'-azinobis(3 ethylbenzthiazoline sulfonic acid) disodium salt (25 mg/ml) in distilled water</td>
<td>0.1 ml</td>
</tr>
</tbody>
</table>

6.5 Buffer for washing plates

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.15M phosphate buffer, pH 7.4 stock</td>
<td>1000 ml</td>
</tr>
<tr>
<td>Tween 80</td>
<td>5 ml</td>
</tr>
<tr>
<td>Physiological saline</td>
<td>9000 ml</td>
</tr>
<tr>
<td>Sodium azide</td>
<td>1 g</td>
</tr>
</tbody>
</table>