DIFFERENTIATION OF BUFFALO FLESH FROM BEEF BY SEROLOGICAL METHODS

By

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THESIS

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DECLARATION

I hereby declare that this thesis entitled DIFFERENTIATION OF BUFFALO MEAT FROM BEEF BY SEROLOGICAL METHODS is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award to me of any degree diploma, associateship, fellowship or other similar title of any other University or Society.

Signature of the candidate

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CERTIFICATE

Certified that this thesis, entitled DIFFERENTIATION OF BUFFALO MEAT FROM BEEF BY SEROLOGICAL METHODS is a record of research work done independently by Sri. E. Somasekharan under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to him.

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INTRODUCTION

INTRODUCTION

The Indian population of 680 million people presents a very complex nature of food habits due to the multiracial characteristics of different ethnic groups. Though 67 per cent of the total population are nonvegetarians the per capita consumption of meat is very low due to socio-economic problems. Nevertheless, the total meat production in India at present is about 829,000 metric tonnes annually of which 189,000 metric tonnes are from cattle and buffaloes. A large majority of our population is religious and sentimental and their food habits and customs are partially based on religious taboos.

A large section of the meat eating population considers mutton and chevon as choice meat and have objections in eating beef because of religious sentiments. In addition to religious taboos, traditions and customs of the society, the availability of meat and wide variations in the price of meats are attributes in the preferential demand of certain meats over others. Partial or total prohibition of slaughter of cows is in force in many states in India. Export of beef is banned in India

by law but there is no restriction on the slaughter of buffaloes and the export of buffalo meat. In Kerala, slaughter of bovines for meat purpose is rampant. Kerala is the market for the unproductive cattle of the neighbouring states and as such, beef is available in plenty compared to buffalo meat and beef production is greater in this state. A section of the population of Kerala has preferential demand for buffalo meat over beef because of various reasons.

Adulteration and misrepresentation are problems encountered in the meat industry throughout the world. It is estimated that 25 to 30 per cent of the edible meat sold in India are adulterated. Substitution of beef for buffalo meat is suspected in the meat export trade and domestic markets in certain parts of the state. Merchandising of meat is a commercial enterprise and so there is always, every incentive to adulterate the meat of choice with other easily available meat, for profit making. Other factors contributing to the adulteration of buffalo meat with beef are, the difficulty in detecting the adulterant due to its closeness to the buffalo meat in physical, chemical and organoleptic characters, and the higher

demand for the latter from the consumers.

Substitution of inferior meat for that of higher quality flesh, eg. beef is a type of fraud commonly encountered in European countries especially in times of scarcity (Kaplan and Buck, 1951). Detection of such deception, is not always easy, especially in comminuted state, or when it is so prepared that the anatomical features are not outstanding (Ginsberg, 1948).

Many a time the regulatory official or the food scientist faces the problems of identifying the meat sample with respect to the species from which it is derived. These types of problems might arise on account of some medico-legal involvement and a piece of flesh might prove to be a valuable material for investigation or it might turn out to be a case of misrepresentation or adulteration (Singh and Yadav, 1962).

Consumer protection is the primary duty of the public health veterinarian and all those who are involved in food regulations. Consumer should get what he wants and for what he had paid. So detection of adulteration turns out to be the most important need in the present context.

Various chemical methods like estimation of glycogen content, Hexabromide value and Iodine value have been employed by many workers in the past for the differentiation of meat of food animals (Ginsberg, 1948; Kaplan and Buck, 1951). The methods to determine the species of origin of meats have not improved very much in the food control due to the difficulties in investigation and lack of proper techniques which are easy, quick and confirmatory.

Many authorities have reported that biological tests based on precipitation, gel diffusion, complement fixation and passive hemagglutination inhibition are suitable techniques for the detection of meat adulteration (Ginsberg, 1948; Kamiyama et al. 1978)[a]. Isoelectric focusing and electrophoresis of muscle and serum protein are other techniques reported for the purpose (Ramadas and Misra, 1974; Abraham, 1982; Keay and Doxy, 1982).

Among the serological tests, double diffusion in agar gel is easy to do, the equipments and chemicals required for the test are cheap and easily available and the results of the test can be preserved for later reference.

Attempts have been made in India by different workers for the differentiation of beef and buffalo meat (Singh and Yadav, 1962; Tagore et al. 1977; Prasad and Misra, 1981). But the difficulty encountered by all of them was the strong cross reaction. Foreign workers have also reported about the difficulties encountered in differentiating meats of closely related species (Proom, 1943; Evans, 1967; Furminger, 1964).

Even though the adulteration of buffalo meat with beef is suspected in Kerala no serological tests seems to have been developed for the differentiation of beef from buffalo meat. Hence the present study was undertaken to suggest easy, quick and reproducible serological test viz. double diffusion test in agar gel and immunoelectrophoresis for the differentiation of beef and buffalo meat. It was also envisaged to study the minimum time required for the development of antibodies in rabbits, determination of the minimum time required to produce an identifiable reaction in double diffusion test and the efficacy of different antigens for the production of precipitin antiserum.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Adulteration of meat is an age old vice and has been reported from developed as well as underdeveloped countries (Ginsberg, 1948; Oswald, 1953 and Pinto, 1961). The practice is in vogue in India because of several reasons like religious taboos, preferential demand for certain kinds of meat and large profit motive of meat traders (Shanmugam and Ranganathan, 1972; Sherikar et al. 1979). Brandley et al. (1966) have stated that meat is subject to adulteration during production, preparation and distribution and as such creates an important control problem. There will also be tendency to substitute other ingredients of lesser cost for meat. Adulteration of beef with horse flesh has been reported by Kaplan and Buck (1951) and Warnecke and Saffle (1968). Jacob (1976) reported that 25 to 30 per cent of the edible meats sold in India are adulterated. Wholesomeness of meat is lost by adulteration. Species identification of meat is necessary for the protection of the consumer's interest. Another reason for differentiation of meat is for enforcement of laws like Equine meat Inspection Act, 1920; of U.S.A. and Anti cow slaughter Act, 1956 of U.P. (India).

Rabbits: as serum raising animal

Proom (1943) reported that rabbits of 2 kg and over are good for serum raising. Evans (1957) recommended rabbits as the best laboratory animal for antibody production. Katsube and Imaizumi (1968) observed that variations may occur in the antisera produced by different species of rabbits immunized alike in all respects. One of the important considerations for immunological investigation is the selection of suitable species of experimental animals. Rabbits were used as experimental animals for antiserum production because of their ability to respond to a variety of antigens, relatively larger volume of antiserum and the moderate space requirement for housing (Crowle, 1973). Garvey et al. (1977) reported that even among rabbits certain strains like Dutch and Newzeland were the best antibody producers.

Immunization and immunising agents

In artificial immunization, selection of reactive animals, type of antigen, route of antigen injection, dose of antigen, and the number and schedule of injections are all important factors (Proom, 1943; Ginsberg, 1948; Warnecke and Saffle, 1968; Kwapinski, 1972 and Libby, 1975).

Dean and Webb (1926) used horse serum as antigen. The first two or three injections were given intravenously followed by subcutaneous or intramuscular route. A tapering dose of 5 ml, 3 ml, 2 ml and 1 ml serum was used. Proom (1943) and Weitz (1952) have recommended intramuscular injection of native serum and alum precipitated serum protein as an antigen to raise antiserum. The work done by Proom (1943) revealed that intra peritoneal injection of alum precipitated serum proteins resulted in the production of antiserum of less specificity and titre while intramuscular or subcutaneous injection gave better results. De Pagondo and Bordner (1943) found that two injections of meat extract by intraperitoneal or subcutaneous routes at two months interval gave good results. Ginsberg (1948) used an extract of meat in saline as antigen. Multiple injections of sterile whole serum by intravenous route was recommended by Kaplan and Buck (1951) to raise antihorse serum while Weinstock (1953) advocated a single injection of alum precipitated horse serum antigen for the production of antisera of greater potency and specificity.

Alcohol precipitated serum protein was used as antigen

by Pinto (1961). He used progressively increasing doses of protein for the three injections and got a maximum serum titre of 1 in 8000 to 1 in 10,000 after the third injection. Singh and Yadav (1962) could get high titre sera using bovine and buffalo plasma as antigen. Furminger (1964) used myosins isolated from various animals to raise precipitin antibodies in rabbits. Six courses of six intravenous injections each were required to raise the antibody to a titerable level and found that the antiserum so produced was highly cross-reacting. Pandey and Pathak (1966) produced rabbit anticow and rabbit antibuffalo serum using alum precipitated bone extracts from these species. They also produced antisera to these antigens by cross immunization of these two species of animals.

Warnecke and Saffle (1968) in a comparative study, raised antisera in rabbits against actomyosin, whole serum, muscle extract in saline and distilled water, and alum precipitated serum and meat extracts. They also used freeze dried beef extract as such and along with Freund's incomplete adjuvant as antigens. Their results revealed that the serum with highest titre was obtained with Freund's incomplete adjuvant while serum raised against alum precipitated muscle proteins from cow and buffalo

gave least titre.

Katsube and Imaizumi (1968) in a comparative study used several antigen viz. serum (raw and boiled) haemoglobin, meat extract (raw and boiled) concentrated meat extract with Freund's incomplete adjuvant and alum precipitated serum proteins. Their results revealed that the antigen with Freund's incomplete adjuvant was effective in raising antisera of high titre and most species specific serum was obtained against boiled serum.

Species specific antisera against mutton and beef were raised in rabbits by intraperitoneal injections of meat extract in saline by Shanmugam and Ranganathan (1977). Sherikar et al. (1979) successfully raised antisera with meat extract in Freund's incomplete adjuvant. Hayden (1979) could raise only nonspecific antisera against myoglobin of different species of animals. Kwapinski(1972) reported that the time required for the antibodies to appear after injection of antigen is variable depending on the nature of antibodies. Precipitins to bovine gamma globulins were detected in rabbits to seven to fifteen days whereas antibodies against BSA appeared in 15 to 29 days. Isolated antigen provoked more easily antibody production than intact cells.

Harvesting and storage of antisera

Pinto (1943) recommended withdrawal of feed to rabbits 24 hours prior to terminal bleeding to ensure a clear serum. Garvey et al. (1977) have reported that for the proper separation of antiserum from the clot, the harvested blood has to be kept at room temperature for two hours and overnight at 4°C in refrigerator.

Storage of antisera

Glenn (1961) observed that rabbit antisera can maintain activity for months when stored aseptically at 4°C or with preservatives. Crowle (1973) reported that freezing is the best method for storing antisera from a few days to few years.

Preservatives of serum

Weitz (1952) reported that all preservatives impart opacity to serum and quickly deteriorated the quality of serum even when stored at 4°C. Merthiolate 0.0001 per cent, sodium azide 0.1 per cent, Phenol 0.25 per cent, a combination of sodium azide 0.05 per cent and acriflavin 0.01 per cent or 0.2 per cent each of ethylparaben and methylparaben can be used as preservatives of antiserum. (Crowle, 1973; Garvey et al. 1977).

Crowle (1973) reported that merthiolate may act against antibodies in the immune serum. Chase (1967) suggested that hemolysis during blood collection for antiserum production is to be avoided, since strong hemolysis resulted in quick enzyme degradation of immunoglobulins on storage.

Cross-reaction in serological tests

Species specificity of antiserum

For the serological identification of animal species of meat, much stress has been given to the preparation of species specific antiserum (Uhlenhuth, 1905; Proom, 1943; Kaplan and Duck, 1951; Ishikawa, 1960; Pinto, 1961 and Katsube and Imaizumi, 1968). Proom (1943) observed cross-reactions between antisera of ox, sheep and goat with antigens of heterologous species from among them.

Pinto (1961) observed cross-reaction between ox, buffalo, goat and deer. He also reported the removal of cross-reacting antibodies by absorption with diluted heterologous sera. Singh and Yadav (1962) experimentally proved that there is close antigenic relationship between buffalo and bovines. Pandey and Pathak (1966) reported

that cow and buffalo bone antisera of rabbit origin reacted giving the same number of precipitin lines with corresponding antigens of either species. Their findings further revealed that antisera produced by cross-immunization with alum precipitated bone extracts, could differentiate not only bone extracts, but even other tissues and secretions and thereby cross reaction could be overcome. Warnecke and Saffle (1968) observed cross-reaction between lamb and beef and succeeded in making the antisera specific by absorption with small quantities of freeze dried antigen. Tagore et al. (1977) rendered antibuffalo and antibovine serum species specific by absorption with heterologous whole sera as recommended by Pinto (1961).

Kamiyama et al. (1978)[b] observed cross reacting antibodies against bovines, sheep and goat and they isolated the species specific fraction of antibody from the antisera by immunoadsorbant chromatography, except for sheep and goat.

Many workers have advocated cross immunization as a suitable technique for the production of species specific antisera. Uhlenhuth (1905) developed cross immunisation to produce species - specific antiserum for differentiation

of serum proteins from ox, sheep and goat. Weitz (1952) cross immunized sheep and goats with each others alum precipitated serum and produced highly species specific antisera when the heterologous antibodies were removed by absorption. Durrand and Schneider (1962) reported that serum with maximum discretionary capacity can be obtained by immunizing closely related species viz. buffalo for raising anticow sera and vice versa. The same technique had been adopted by Pandey and Pathak (1966) in India for raising antisera of high specificity. The method adopted by Litchter and Bray (1964) for the production of antisera of highest specificity was the isoimmunization where antigens were given to the same species of animals like rabbit tissues to rabbits. Specific antigenic fractions separated by zone electrophoresis were used by many workers for immunizing animals (Croisille, 1962; Laron and Assa, 1962).

Hayden (1979) failed to remove the cross reacting antibodies from antilamb myoglobin sera against bovine myoglobin by absorption. Sherikar *et al.* (1979) reported that inspite of cross-reactions, meats of related species could be identified by calculating the diffusion coefficient

in double diffusion test. Immunoelectrophoresis was used for identification of meats from different animal species by Ramadass and Misra (1981).

Absorption of antisera

According to Landsteiner (1947) absorption of heterologous antibody needs very high titre antisera and this technique is not uniformly successful. Kubota (1967) reported that antiserum against boiled serum was specific against alkaline extracts of cooked beef or pork, mutton, horse meat and chicken. According to Weitz (1952) the absorption of heterlogous antibodies in antiserum will not affect the titre of homologous antibodies and as such, by absorption nonspecific antisera can be made specific with heterologous sera. Hafeze et al. (1961) removed cross reacting antibodies by dilution of antisera to 1:1000. Pinto (1961) used one part of 1:200 dilution of normal heterologous serum for absorption of cross reacting antibodies from antisera against ox, buffalo and deer. Crowle (1973) reported that production of monospecific antisera was usually impossible without special immunization or purification techniques, but these may be more complicated than making polyspecific antiserum, monospecific, by

absorbing out the unwanted antibodies and in many immunological works this absorbed serum was sufficient. Prasad and Misra (1978) used rivanol precipitated antisera for identification of meats and it was seen that rivanol treatment produced complete loss of specificity even for the homologous antigen.

Titre of antiserum

Cromwell (1925) reported that the point of disappearance of recognisable precipitation on increasing the dilution of the antigen does not give a direct evidence of the strength of the antiserum. A good precipitating antiserum should show a very definite ring formation when layered at the bottom of a 1:8000 dilution (Proom, 1943) and 1 in 800 (Libby, 1975) of antigen and no reaction with a 1:50 dilution of sera of unrelated species. According to Oswald (1953) a satisfactory antiserum for the purpose of meat identification will give a strong precipitation at a dilution of antigen 1:1000 within 20 minutes at room temperature and will not cross-react with antigen of other species. Pinto (1961) judged the potency of antiserum by the intensity of precipitation reaction with 1:2000 dilution of antigen rather than positive reaction

at higher dilutions.

Tests for identification of meats of various species of animals

Physical, chemical and biochemical tests have been reported as valuable tools for the identification of meats from different species of animals (Edelmann et al. 1943; Crowell, 1944; Guptha and Hilditch, 1951 and Hynds, 1951). Though tests like glycogen tests, hexabromide value, linoleic acid content, Iodine value and refractive index have been employed for identification of meats by the above workers, such tests have limitation in the successful identification of meats, since they are neither specific nor wholly conclusive (Edelmann et al. 1943).

Serological techniques

Tube precipitation:

The discovery of precipitin antibodies and the precipitin tests by Krause (1897) opened the path for numerous applications for the test like study of proteins and their linkages to other materials. He showed that the contact or mixing of clear antisera with clear solutions of antigen gave rise to the turbidity or precipitate.

These precipitins were quite specific, but group precipitation takes place when related proteins are treated with a serum homologous to one of them. Crowle (1973) and Garvey *et al.* (1977) described precipitation as an immunological reaction occurring at the combination of an antibody with its corresponding antigen resulting in a visible aggregation.

Uhlenhuth (1901) demonstrated that precipitation test could be used to determine the species origin of blood and muscle tissue.

Biological precipitation test has been described as an important, reliable and superior control procedure for identification of adulteration by Edelmann (1943), Kaplan and Buck (1951) and Weinstock (1953). According to Ginsberg (1948) the precipitation test could be carried out without any difficulty even in abattoir laboratories because of its simple technique.

Penso (1955), Pandey and Pathak (1962) and Shanmugam and Ranganathan (1979) have described precipitation test as a simple advantageous method which could be applied to fresh, dried, pickled and salted meats for their identification.

Gel diffusion test

Gel diffusion test is the most widely accepted method for the identification of different meats. (Van den Heever, 1962; and Warnecke and Saffle, 1964). In the double diffusion technique originally described by Ouchterloney (1940) antigen and antibody in solution were placed in opposing wells cut in agar gel and allowed to diffuse through the agar medium. The line of precipitate was formed where the antigen and antibody meets at optimal proportions (Baker and Breach, 1980). According to Maurer et al. (1963) and Merchant and Packer (1967) the number of precipitation bands developing in a double diffusion test usually can be interpreted as representing the minimum number of precipitating systems present. In Ouchterloney's double gel diffusion tests, gradients of both reactants, namely antibody and antigen, formed from the two opposing wells, gave rise to the precipitation line at the appropriate dilution. The zone of precipitation shifted slightly towards the weaker reactant (Crowle, 1973 and Sherikar et al. 1979).

Fugate and Penn (1971) reported that immunodiffusion in agar gel was a relatively rapid and simple test.

Crowle (1973) reported that the adaptability of double diffusion tests to compare antigen preparations makes these tests versatile in contending with such problems.

Thornton and Gracey (1974) reported that it can be applied to meats heated up to 80°C for 10 minutes. According to Tagore et al. (1977) the gel diffusion test can enumerate the minimum number of antigen antibody systems involved in the reactions at a given time, besides providing a permanent record of the test.

According to Garvey et al. (1977) the main advantages of double diffusion lie in the detection of more than one antigen-antibody system and high degree of sensitivity, the lower level of detection being a few micrograms of antigen and antibody.

Double diffusion in agar gel had been successfully employed by several workers for identification of meats from different animals. Vanden Heever (1962) reported that agar gel diffusion technique can be used to identify meat from sheep, cattle, and horses. He also proved that this could be applied successfully on 'biltong' (the sun dried flesh) for identification.

Furminger (1964) using gel diffusion tests showed that a high degree of cross reaction existed between myosins of the species within the same class of animals. He studied the cross-reactivity of pig, sheep, horse and bovine myosins by Ouchterloney's plate technique and proved that species specificity of myosins of these species cannot be used to differentiate between muscles of these species.

By employing agar gel diffusion test, Furminger (1964) showed that cross-reaction existed between members of the bovidae family while, antihorse, antipig, and antidog sera showed high specificity. On comparison of meats subjected to cooking at 90°C for 20 minutes, and 30 minutes the former gave a diffuse precipitation line in gel diffusion while the latter did not develope any line of precipitation with homologus antigen. He has also confirmed the finding of Vanden Heever that the sun dried meats could also be identified with gel diffusion test.

Pandey and Pathak (1966) reported that by gel diffusion test, they could distinguish tissues including bones from bovines and buffalo when tested against antisera raised with bone extract by cross immunization of cow and buffalo

and the antiserum raised in rabbits failed to do so.

After absorption of cross reacting heterologous antibodies from homologous immune sera, raised in rabbits, Warnecke and Saffle (1968) could identify horse meat, pork, lamb and beef by agar-gel diffusion test. Mendes and Ribeiro (1971) reported that they could satisfactorily differentiate bovine, equine, swine, dog and cat meats by agar gel diffusion tests. The antisera were raised in rabbits against alum precipitated whole serum from these animals.

Successful identification of beef from buffalo have been reported by Tagore et al. (1977) by using absorbed antisera in the gel diffusion test.

Hayden (1979) tested antisera to myoglobin from various meat animals to detect the addition of flesh from these animals in ground beef, by gel diffusion test and he could detect adulteration of beef with pork and lamb up to 3 per cent. Presence of the meats in sausages heated up to 70°C was detectable by double diffusion in agar gel. Sherikar et al. (1979) reported that the differentiation of meats from bovines, buffalo, sheep, goat, pig and poultry was possible by Ouchterloney's

double gel diffusion tests, in spite of cross reaction with heterologous antibodies in immune sera between related species, by calculating the diffusion coefficients.

Media for gel diffusion

According to Crowle (1973) immunodiffusion media include, agar, agarose, cellulose acetate, polyacrilamide and gelatin. Agar is most popular among workers due to reasons like cheapness, wide availability, inertness to reactants, ease to wash off excess reactants, and transparency of gel. It is easy to cut wells or trough in the gel and their shape is retained. Crowle (1973) suggested that one per cent agar in normal saline was most suitable for gel diffusion tests. Eventhough the gel can be used immediately after it has solidified, some workers have recommended the ripening of the gel for several days for accelerating the immunodiffusion reaction (DeCarvalho, 1960). Williams and Chase (1971) stated that the advantage of immunochemical analysis in gels over many physicochemical methods was the former's higher sensitivity and greater resolving power.

The results of gel diffusion tests were recorded in numbers of precipitation bands forming, their position

between reactant wells, whether they are sharp and well defined, or are fuzzy and whether the lines confluent, whether there is spur formation or the lines cross (Crowle, 1973).

The time lapse preceeding visible precipitation in the double diffusion test is controlled by three main factors. The first two factors govering the diffusion rates of the reactants are their diffusion coefficients and absolute concentration. The third factor is how soon the antigen and antibody forms visible complexes after their fronts have met (Aladjem et al. 1959; Aladjem, 1964; Jameson, 1964). The concentration of the antigens and antibodies, the distance between their sources, temperature, agar concentration and gel additives are other factors which influence the formation of precipitin bands (Wilson and Pringle, 1954). If the reactant sources are too far apart, antigen and antibody fronts may meet but the precipitation bands may not appear due to lack of concentration of reactants (Aladjem et al. 1959). They also reported that when other factors were held constant the visible precipitation sensitivity of double diffusion test for a given system was directly proportional to the

closeness of the antigen and antibody depots.

Immunoelectrophoresis

Immunoelectrophoresis, a technique for the study of antigens and antibodies is based on two of their properties, ability to precipitate on agar gel with specific antibody and antigen respectively (immunodiffusion) and their characteristic mobility in an electric field (electrophoresis)(William and Garbar, 1955). The solution to be examined for antigen or antibody, is first separated into its componants by electrophoresis in agar or agarose gel on a microscope slide (Scheidegger, 1955). At the end of the electrophoretic separation the protein fractions might have moved through the gel, a distance characteristic for that protein. After completion of the run these separated componants were allowed to react with the specific antibodies contained in the antiserum placed in the central trough cut in the gel parallel to the path of the migration of the componants. The antigen componants separated on the basis of their electrophoretic mobilities diffuse from their points of migration, meet and precipitate with the antibodies that have diffused from the trough (Garvey et al. 1977). The

superiority of immunoelectrophoresis over gel diffusion was the higher resolving power of the former (Holland et al. 1962; Dodin, 1963 and Garvey et al. 1977).

Durrand and Schneider (1962) and Sinel and Mentz (1969) have reported successful identification of meats from cattle, horse, donkey, sheep goat and pig by immunoelectrophoresis. Successful identification of human, guinea pig and rabbit serum by the use of immunoelectrophoresis have been described by Makonkankeyoon and Haque (1970).

According to Ramadass and Misra (1981) meats of bullock, buffalo, goat, sheep, pig and chicken could be differentiated by immunoelectrophoresis.

By rocket electrophoresis of specific antibody fractions from antisera against cattle, swine, horse, dog, cat and goat-sheep group Japanese workers Kamiyama et al. (1978)[b] identified different meats up to 0.5 to 1 per cent in concentration.

Electrophoresis and Isoelectric Focussing

These methods though not serological, are gaining importance in the identification of meats from different

animals. Babiker *et al.* (1981) reported that serological methods are less effective to detect horse flesh, when applied to heat processed products. The inherent difficulties due to cross reaction can be overcome by these methods. Dilworth and McKenzie (1970); Mattey *et al.* (1970); Kaiser *et al.* (1980)[a] and Babiker *et al.* (1981) have applied electrophoresis for identification of meats of different species of animals. Mattey *et al.* (1970) could identify rabbit and ox meat heated up to 120°C for 3 to 6 minutes and have reported that this method could be used to analyse unknown mixtures of meats heated up to 25 minutes to such temperatures. Babiker *et al.* (1981) differentiated beef and horse meat heated to varying temperature from 60°C to 120°C for 20 minutes by electrophoresing myofibrillar proteins of these animals.

In India Prasad and Misra (1981) reported that electrophoregrams of muscle esterase enzymes of different species of animals indicated that each age and sex groups possessed species specific enzyme patterns.

Keay and Doxy (1982)[a&b] electrophoresed serum samples from both young and adult cattle, sheep and horses on

MATERIALS AND METHODS

MATERIALS AND METHODS

Rabbits

For the purpose of raising immune sera 26 rabbits of both sexes weighing about one kilogram were procured from the Small animal breeding station attached to the College of Veterinary and Animal Sciences, Mannuthy. The rabbits were divided into six groups at random for immunization as per the programme given below.

Group	No. of rabbits	Antigen used
1	6	Buffalo meat extract in normal saline
2	6	Buffalo meat extract in distilled water
3	3	Buffalo meat extract with Freund's incomplete adjuvant
4	3	Alum precipitated buffalo meat extract
5	4	Beef extract in saline
6	4	Beef extract in distilled water

The rabbits were kept under observation for one week before immunisation until they get acclamatised to the new environment. Individual rabbits in each group

were identified by ear punch marks. They were fed with soaked bengal gram and green grass ad libitum.

Preparation of antigens

Meat samples

All the meat samples for the preparation of antigen for immunization and for immunodiffusion tests were collected from the Municipal slaughter house, Kuriakhira, Trichur, except dog meat which was procured from various departments in the college.

Preparation of buffalo meat extract in saline

The methods described by Merchant and Packer (1967) were adopted for the preparation of meat extract antigen in saline. Thirty grams of fresh buffalo meat was cut into small pieces and blended in 50 ml of normal saline solution. The mixture was kept over night in refrigerator at 4°C with occassional stirring. Next day the mixture was filtered through muslin cloth. The filtrate was collected and centrifuged at 3000 rpm. for 10 minutes. The supernatant was collected and used as antigen for immunization.

Beef extract in normal saline

The same procedure was adopted for the preparation of beef extract in normal saline and used as antigen.

Buffalo meat extract in distilled water

The procedure adopted for the preparation of buffalo meat extract in distilled water was the same as described for the preparation of buffalo meat extract in saline except that distilled water was used instead of normal saline.

Beef extract in distilled water

This was prepared in the same manner as described for the preparation of buffalo meat extract in distilled water.

Alum precipitated buffalo meat extract

The method described by Garvey et al. (1977) was adopted in this study for the preparation of alum precipitated meat extract. Fifty grams of meat was blended with 100 ml of distilled water in an electric blender. This meat slurry was kept overnight in refrigerator at 4°C. The slurry was sieved through sterilised muslin cloth to remove the bulk and the filtrate was collected

and centrifuged at 3000 rpm for 30 minutes. The supernatant was collected and filtered through Whatman No.I filter paper and collected the filtrate. Fifty ml of this filtrate was taken in a beaker and added 22.5 ml of one molar sodium bicarbonate solution. This mixture was kept stirred when 50 ml of 0.2 molar aluminium potassium sluphate in phosphate buffer saline solution was added. Care was taken to avoid frothing and kept undisturbed for 15 minutes. This was centrifuged at 3000 rpm for 30 minutes. The precipitate obtained was washed thrice in phosphate buffered saline by centrifugation and used as antigen after reconstituting in saline. The precipitated protein from 10 g of meat was reconstituted and given as a single dose.

Buffalo meat extract with Freund's incomplete adjuvant

The buffalo meat extract was prepared in the very same manner as described for the preparation of alum precipitated meat extract. Equal quantities of meat extract and Freund's incomplete adjuvant (2 ml of each) were taken in a test tube and mixed well by agitation with a cyclomix until a stable water in oil emulsion

was formed. The stability of the emulsion was tested by placing a small drop of the blended product over water taken in a petri dish. Two ml portion of this stable emulsion was used as the antigen for raising antisera in rabbits by intramuscular injection.

Antigen for absorption of cross reacting antibodies in the serum

Thirty grams of fresh beef was cut into very small pieces and blended in 50 ml of normal saline. The mixture was kept over night in the refrigerator at 4°C. Next day this mixture was filtered through three layers of muslin cloth and collected the filtrate. The filtrate was vialled in 2 ml ampoules and freeze dried. This was used for the absorption of cross reacting antibodies in antibuffalo sera.

The buffalo antigen for absorption of cross reacting antibodies was also prepared in the same manner.

Antisera to beef and buffalo extract antigen in distilled water and normal saline

Six rabbits belonging to group 1 were immunized against buffalo meat extract antigen in normal saline

and six rabbits of group 2 were immunised with buffalo meat extract antigen in distilled water. The rabbits in group 3 numbering four were immunized with beef extract antigen in normal saline and the four rabbits in group 4 with beef extract antigen in distilled water.

Schedule of immunisation of rabbits

Group No.	No. of rabbits in the group	Antigen used.	Route of administration.	Interval between injection.	Dosage
1	6	Buffalo meat extract in normal saline solution	Intraperitoneal	4 days	1st injection of 3 ml and subsequent 9 injections of 5 ml
2	6	Buffalo meat extract in distilled water	-do-	-do-	-do-
3	4	Beef extract in normal saline solution	-do-	-do-	-do-
4	4	Beef extract in distilled water	-do-	-do-	-do-
5	3	Alum precipitated buffalo meat protein	Intramuscular	7 days	Meat protein from 10 ml of meat extract

Group No.	No. of rabbits in the group	Antigen used.	Route of administration.	Interval between injection.	Dosage
6	3	Buffalo meat extract in Freund's incomplete adjuvant	2 intra muscular injections followed by 3 injections subcutaneously	7 days + daily	1 ml of meat extract with 1 ml adjuvant.

Injection of antigen

The rabbits were held by the attendant with its back resting on the table, exposing the ventral area completely. The hind portion of the rabbit was elevated slightly for the intestines to move towards the diaphragm. The fur on the abdomen was wetted with rectified spirit, slightly caudad to the center, exposed a small portion of the skin by brushing aside the fur. The needle on the loaded syringe was introduced into the peritoneal cavity, through the exposed skin. Penetration of the peritoneum was indicated by cessation of resistance against the needle. Retracted the needle

slightly to reduce the possibility of puncturing the intestines. Piston of the syringe was withdrawn slightly to confirm that intestines were not punctured. The site of injection was shifted from left side to right side for every alternate injection to reduce trauma.

The antigen injections were given using a sterile glass syringe with 20 SWG hypodermic needle. In all cases, an initial dose of 3 ml of the respective antigens were given. The dosage was revised to five millilitres from second injection onwards.

The antigens were given every 4th day and a total of 10 injections were given to all the animals as shown in the schedule.

Test bleeding

Test bleeding by scarifying the ear was done and the potency of the serum thus collected was tested by gel diffusion in all cases.

Terminal bleeding

After the 10th injection the rabbits were given 10 days rest and terminal bleeding was done by severing

the carotid artery after mechanical stunning. Twenty four hours prior to final bleeding, feed but not water, was withdrawn to get clear serum. Care was taken to avoid hemolysis.

Preparation of immune sera using alum precipitated muscle protein

Alum precipitated meat protein was used to raise immune sera only in the case of buffalo meat. The alum precipitated protein from 10 g of buffalo meat was reconstituted with 2 ml of normal saline. All the animals in group 5 was given 2 ml of the protein suspension intramuscularly. The injections were repeated at weekly intervals for six weeks. The test bleeding was done to determine the titre of the antibodies in the serum on every alternate day from 7th day. After the sixth injection the animals were sacrificed and collected the blood for preparation of antiserum.

Immune sera against buffalo meat extract with Freund's incomplete adjuvant

The method of immunization suggested by Crowle (1973) was followed. Two injections (2 ml of the antigen) were

given 7 days apart intramuscularly. The animals were given rest up to 34th day. The rabbits in this group were given 3 ml each of the buffalo meat extract subcutaneously on 35th, 36th and 37th day after the first injection. The rabbits were given rest up to 52nd day and then sacrificed and collected the blood.

The test bleeding was done on the 7th day, and every alternate days till the development of observable levels of antibody as determined by gel diffusion test.

Collection and preservation of antiserum

Terminal bleeding of the rabbits was done by severing the carotid artery after stunning. The method described by Garvey et al. (1977) was adopted for the preparation and preservation of antiserum. The freshly collected blood was allowed to stand at room temperature for 1-2 hr for clot formation. Carefully detached the clot from the wall of the test tube using a sterile glass rod and stored at 4°C in the refrigerator overnight for contraction of the clot. Decanted the separated serum in the test tube and clarified by centrifuging at 3000 rpm for 10 minutes. The sera from the different animals were pooled together

group wise. Merthiolate was added to the final concentration of 0.0001 per cent as preservative. The serum was transferred to small vials, stoppered and stored at 4°C in refrigerator.

Absorption of cross reacting, heterologous antibodies

To absorb the heterologous cross reacting antibodies in the antiserum, the method suggested by Warneck and Saffle (1968) was employed. The buffalo antiserum was absorbed with freeze dried beef extract. Equal quantities of antiserum and heterologous antigen (to 5 ml of antibuffalo meat serum, freeze dried meat extract from 5 ml of beef extract) were mixed and allowed to stand at room temperature for two hours and kept in the refrigerator at 4°C overnight with occassional stirring. Then the mixture was centrifuged at 3000 rpm for 15 minutes and the supernatent was collected in sterile vials.

Antibeef serum was absorbed with freeze dried buffalo antigen in the same manner.

Preparation of test antigen

The test antigens from various animals for doing gel diffusion test were prepared from skeletal muscles in

normal saline. Ten gram of meat was blended with 15 ml of normal saline, kept overnight at 4°C in refrigerator, with occassional stirring, and filtered and vialled. The antigens were made afresh every time for use. Test antigens were prepared from 50 samples each of buffalo meat and beef, 25 samples each of mutton and chevon and 10 samples each of pork and dog. In immunodiffusion tests these antigens were used as such and after dilution to 1 in 10 in all the tests.

Preparation of gel for gel diffusion test

One gram of Difco noble agar was added to 100 ml of normal saline in a conical flask and melted the agar by keeping the flask in a water bath at 100°C for 30 minutes. When the agar was completely melted the same was poured into precoated petri dishes of 7.5 cm diameter. The coating was done by swirling some melted agar in the petri dishes and discarding the excess. The agar in the petri dish was allowed to stand at room temperature for an hour and then transferred to the refrigerator at 4°C.

Cutting of wells

A template with markings for the wells, one in the

center and six around it, all equidistant from each other, 5 mm apart and of 3 mm diameter, was placed underneath the petri dish with the solidified gel and the pattern was marked in the gel with a glass tube having 3 mm outside diameter. The gel in these marked wells was removed by aspiration with a pasteur pipette. Two such sets of wells were prepared in one petri dish.

Gel diffusion test

In the gel diffusion test the antibuffalo serum and antibeef serum raised with different kinds of antigens were put to test. Both whole and absorbed antisera were tested in all cases.

The antigen prepared from the different samples of mutton, chevon, pork and dog meat were tested with unabsorbed antibuffalo serum prepared against buffalo meat extract in Freund's incomplete adjuvant.

Fifty samples each of buffalo meat and beef were used for the identification trials.

Two sets of wells were cut in each gel plate, set 1 and 2. The antigen wells in the periphery were numbered from one to six in the clockwise direction from 1'o clock

position. The double gel diffusion test were conducted by the Ouchterloney's method (1949). Gel plates in petri dishes were charged with antisera in the central wells and different antigens in wells 1 and 2 and 5 and 6. The wells 3 and 4 were always used as controls with saline in them. In tests with beef and buffalo antigens and their homologous antisera the schedule of filling antisera was as follows:

Plate No. I

Unabsorbed antibuffalo sera in the left set and absorbed antibuffalo sera to distilled water extract in the right set.

Plate No. II

Unabsorbed and absorbed antibuffalo sera against meat extract in normal salines in set No. 1 and 2 respectively.

Plate No. III

Absorbed and unabsorbed antibuffalo sera in the two central wells in sets 1 and 2 respectively against meat extract in Freund's incomplete adjuvant.

Plate No. IV

Absorbed and unabsorbed antibeef sera against alum precipitated meat extract in the central wells of set 1 and 2 respectively.

Plate No. V

Unabsorbed and absorbed antibeef sera against meat extract in distilled water in the central wells of set 1 and 2 respectively.

Plate No. VI

Unabsorbed and absorbed beef antisera against meat extract in saline in central wells of the set 1 and 2 respectively. In all these cases the test antigen were used as follows:

Well No. 1	undiluted buffalo antigen
Well No. 2	diluted (1 in 10) buffalo antigen
Well No. 3 & 4	Normal saline
Well No. 5	diluted (1 in 10) beef antigen
Well No. 6	undiluted beef antigen

In studies with mutton, chevon, pork and dog meat, in all cases the central wells were charged with anti-buffalo sera against meat extract with Freund's incomplete

adjuvant.

The peripheral wells No. 1 and 2 were charged with undiluted and diluted (1:10) of buffalo antigen, 3 and 4 with normal saline and 5 and 6 with diluted (1:10) and undiluted antigens from mutton, chevon, pork and dog meat.

Differentiation of buffalo meat from beef by immunoelectrophoresis

The technique described by Zewigh and Whitaker (1967) was followed in this work for the immunoelectrophoresis.

Apparatus

1. Electrophoretic tank with electrodes and terminals
2. Power pack with 500 volts D.C. output and 150 mA capacity
3. Connecting wires with clips
4. BOD incubator

Chemicals and reagents

Buffer for electrophoresis

Barbital sodium, mol wt. 206.18-$C_8H_{11}N_2NaO_3$	: 8.5 g
1. N. Hydrochloric acid	: 8.9 ml
Distilled water to	: 1000 ml

Adjusted to pH 8.4

Composition of Phosphate buffered saline pH 7.2 & 0.15 M

Sodium chloride	: 8.00 g
Potassium chloride	: 0.2 g
Sodium dihydrogen (ortho) phosphate	: 0.20 g/l
Potassium dihydrogen phosphate	: 0.2 g/l

Dissolved in 1000 ml of distilled water

Amidoblack stain

Amidoblack 10 B	: 1.00 g
Sodium acetate - acetic acid buffer 0.2 M pH 3.6	: 1000 ml

Decolouriser No.1

Methyl alcohol (absolute)	: 45 ml
Glacial acetic acid	: 10 ml
Distilled water	: 50 ml

Decolouriser No.2

Ethyl alcohol (absolute)	: 40 ml
Glacial acetic acid	: 10 ml
Distilled water	: 50 ml

Materials

Agarose for electrophoresis - Sisco Laboratories, Bombay : 1.00 g

Noble agar - Difco Laboratories (Detroit, U.S.A.) : 0.5 g

Fresh Microscope glass slides 7.5 x 2.5 cm (Blue star) : 50 nos

10 ml glass pipette to deliver hot agarose

Conical flask (Corning) 250 ml : 3 nos

Filter paper (Whatman No.1) : 5 sheets

Metal tube with 2 mm outside diameter

Razor blade

Precoating of glass slides for immunoelectrophoresis

Fresh ordinary microscope slides of the size 7.5 x 2.5 cm were coated by dipping in hot 0.5% agar solution and dried. The dried slides were stored for future use.

Preparation of gel for immunoelectrophoresis

One gram of agarose (Sisco Laboratories, Bombay) was mixed with 50 ml of normal saline in a conical flask over boiling water bath, and finally boiled over the burner directly. To this was added 50 ml of hot barbital

buffer and mixed well.

Preparation of slides for immunoelectrophoresis

The precoated slides of 7.5 x 2.5 cm were placed on level surface. Pipetted 3 ml of hot agarose solution into each microscope slide and allowed to set to get 1.5 mm thick gel.

Punching of wells and trough in the gel

In the center of the solidified agar a trough having 2 mm width 5 cm length was cut with a razor blade parallel to the long axis and the gel, was left undisturbed in this area. On either side of the trough, at one end two wells of 2 mm diameter werecut using a metal tube of 2 mm outer diameter. The agar plugs in the wells were removed by aspiration.

Immunoelectrophoresis

The electrophoretic tanks were filled with the barbital buffer up to the mark. Then the antigen wells in the agarose slides were filled with 0.05 ml of respective antigens using capillary tubes. Twenty samples each of buffalo meat and beef were tested by immunoelectrophoresis and 10 samples each of mutton, chevon, pork

and dog meat.

The manner in which the antigens were charged in different slides is given below.

1. Buffalo meat extract and beef extract.
2. Buffalo meat extract and mutton extract.
3. Buffalo meat extract and chevon extract.
4. Buffalo meat extract and pork extract.
5. Buffalo meat extract and dog meat extract.

All the slides were labelled for identification.

Electrophoretic run

The labelled slides after charging with antigen, four slides were run at a stretch. These slides were placed on the support of the electrophoretic tank and connected to the buffer solution in the electrophoretic tank with soaked filter paper wicks at both ends of the slides. Care was taken to see that the wicks were having proper contact with gel.

The power pack was switched on an hour before the experiment for stablization. After placing the charged slides before starting the run a small marking was made on the gel close to the antigen wells. Then the run was

started by establishing the connections of the power pack to the tank and the buffer and the gel from negative to positive. The tank was closed with its cover. The power out put was set at 150 volts and 12 mA (@ 3 mA/ slide). The run was continued for an hour till the antigens had moved at least 80 per cent of the long axis of the slide (about 4 cm). This was observed by the movement of the small drop of amidoblack on the slide, in level with the antigen well. The supply was cut off and the slides were removed carefully. The precut gel in the center of the slides were removed cautiously and charged with 0.2 ml of the respective antiserum. The charged slides were replaced on the support in the tank, closed the lid and allowed to stand for 24 hours for the immunodiffusion and precipitation to take place.

Washing and drying of electrophoregram

The slides were then transferred from the electrophoretic chamber and washed with two changes of normal saline, the first for six hours and the second for 24 hours. After removing the unwanted protein by washing the wells and trough, slides were dried incubator. Before keeping in incubator the slides were covered with the

moistened filter paper, leaving the trough alone uncovered.

Staining and mounting

The dried slides were placed on level surface and flooded with amidoblack stain and allowed to stand for five minutes. Poured off the stain and rinsed with decolourising solution No.1 three times to get the background clear and the precipitation bands distinctly stained. Then washed with destaining solution No.2 for a few seconds. Dried the slides under fan and mounted with canada balsm.

Experiment with absorbed serum samples

The electrophoresis of 20 samples of beef and buffalo meat and 10 samples of mutton and chevon were also done in the same manner using absorbed antibuffalo serum, instead of unabsorbed serum.

Test to determine minimum time required for the formation of a detectable antigen antibody reaction by gel diffusion

In this experiment the antibuffalo sera raised against the four different antigens viz.

1) meat extract in distilled water

2) meat extract in saline

3) meat extract with Freund's incomplete adjuvant and

4) alum precipitated meat protein were used.

Undiluted and diluted (1:10) buffalo meat and beef extract antigens were tested. The central well was charged in each case with one of the antisera and the wells 1 and 2 charged with buffalo meat antigen, well No. 3 and 4 with normal saline solution and 5 and 6 with beef antigen. Plates were transferred to the refrigerator after one hour of charging and observations made every 30 minutes after the third hour for the development of the precipitate. The test was repeated for 10 meat samples.

RESULTS

RESULTS

Rabbits

Among the 26 rabbits used for raising antiserum in the course of the study 23 rabbits withstood the full course of immunization. Two rabbits died due to haemorrhagic tracheitis. One of the rabbits in the group which received injection of alum precipitated meat extract developed paralysis after the second injection and hence sacrificed. Among the 23 rabbits which withstood the full course of immunization all except one responded by developing detectable levels of antibodies.

The response of the rabbits to the various antigens used for raising antibodies and the titre of antisera produced are summarised in table 1. Among the six rabbits which received injections of buffalo meat extract in normal saline, one did not develop antibodies even after the receipt of eight injections. The immunization of that rabbit was discontinued since it did not respond to the antigen.

The antisera produced from all the rabbits could be effectively used though they showed variations in the titre.

Development of antibodies in the rabbits

The results of the test bleedings done on the rabbits to detect the time requirement for the development of detectable level of antibodies are given in table 2.

The results of the test bleeding from the 7th day to 25th day of immunization of rabbits are shown in table 2. When the rabbits were test bled on the 7th day after the first injection, none of them were found to have developed antibodies as evidenced by negative results in gel diffusion test with meat antigen. From the subsequent test bleeding it was found that the groups of rabbits receiving different types of buffalo antigens developed antibodies within 15 days, whereas the groups receiving beef antigens developed antibodies only between 21 and 25 days. Among the rabbits which received buffalo antigens, the group which received meat extract in distilled water showed the earliest development of antibodies, on the 11th day of initial injection. They had received three injections by that period. The group of rabbits which received buffalo meat extract in saline showed detectable levels of antibodies in their serum on the

13th day (after the 4th injection). The alum precipitated buffalo meat extract and meat extract with Freund's incomplete adjuvant took the longest period of 15 days to evoke detectable level of precipitin antibodies in the test bled serum.

The earliest time for the development of antibodies in the rabbits was 21 days in the group which received injections of beef extract in saline. This group had received six injections of antigen. The maximum of 25 days observed for the development of antibodies was in the group of rabbits which received seven injections of beef extract in distilled water.

The efficacy of various antigens viz. alum precipitated meat extract, meat extract in incomplete Freund's adjuvant, meat extract in distilled water and meat extract in saline solution to produce precipitin antibody in rabbits was determined by comparing the intensity of precipitin bands and time required for their appearance in the gel. The observations on these parameters are summarised in table 3. In the present study it was found that the antisera against buffalo meat extract with Freund's incomplete adjuvant gave precipitation lines with maximum

intensity and clarity, when tested with undiluted and diluted (1:10) buffalo meat and beef test antigens alike at 12 hours and 18 hours after charging of the reactants. The antisera against meat extracts in distilled water gave precipitin lines of less intensity compared to the lines given by antisera produced against antigen in Freund's incomplete adjuvant within the same time intervals after charging.

The precipitin bands produced by antibuffalo serum raised against meat extract in saline to the undiluted test antigens of buffalo meat and beef were less defined at 18 hours when compared with the bands produced by antisera raised against meat extract in distilled water and meat extract with Freund's incomplete adjuvant. When the diluted buffalo meat and beef test antigens were tested the former produced a faint line of precipitation at 12 hours while there was no line of precipitation in case of the latter.

The antisera raised against alum precipitated buffalo meat protein gave clear, but thin precipitation bands at 18 hours of charging, to all antigens. Faint precipitation bands were observed at 12 hours with dilute

buffalo antigen. Similar bands were observed at 18 hours with undiluted beef antigen. Though there were no precipitin band formation at 12 hours with diluted beef antigen faint line of precipitation was observed with undiluted beef antigen.

Minimum time required for the appearance of visible precipitation bands in the gel diffusion test

In a study to determine the time lapse from charging the wells with the reactants to the appearance of precipitation lines, it was revealed that the antisera raised against buffalo meat extract in Freund's incomplete adjuvant required five hours in all the 10 different undiluted buffalo meat and beef antigens.

When the experiment was repeated with antibuffalo sera raised against meat extract in saline, distilled water and alum precipitated meat protein, the minimum time required for the appearance of visible precipitation reaction was observed to be five hours when tested against undiluted buffalo meat and beef antigen.

Results of gel diffusion tests to differentiate buffalo flesh from beef

a) Antibuffalo serum against buffalo meat extract in distilled water

Antisera collected on the 11th day, 28th day, 35th day and on terminal bleeding were tested with beef and buffalo meat antigens (both undiluted and diluted : 1:10). The precipitation bands were produced in all the tests within 18 hours. The band produced by buffalo meat antigen was thick. The band in case of beef was identical to that of buffalo meat. The precipitation lines produced by both beef and buffalo meat showed complete identity with the absence of spur formation or intersection. These sera were tested with all the 49 samples of buffalo meat and beef and got the same pattern of results consistently as above. The antisera harvested on the 11th day gave one broad band each with buffalo meat as well as beef antigens within 18 hours but were fuzzy. The sera from the other two test bleedings and terminal bleedings gave well defined and thick bands, within 12 hours identical in all respects, to beef as well as buffalo meat antigens.

Absorbed antibuffalo serum

When the antibuffalo serum against meat extract in distilled water was tested with buffalo meat and beef antigens in gel diffusion test, no line of precipitation was observed in both cases until sixth day of charging the wells. A very faint line of precipitation was observed on the 7th day in the case of both beef and buffalo meat. Similar results were observed with undiluted as well as diluted (1 in 10) antigens from all the samples when tested with absorbed antibuffalo serum. When the tests were repeated with absorbed sera on the 35th day and of terminal bleeding no differences in the results were observed (Fig. 1).

b) Tests with antibuffalo sera against meat extract in normal saline solution

The antisera against buffalo meat extract in normal saline collected during early periods of immunisation viz. 13th day and 21st day, gave precipitin bands in double diffusion test in agar gel. Positive precipitin bands appeared within 18 hours in the gel against homologous antigen as well as beef antigen. There were one thick line and a thin line of precipitation against both

antigens in both dilution. When this serum was absorbed with beef and tested, it did not give any reaction against both the antigens.

The serum collected on terminal bleeding when tested gave precipitin bands within 12 hours to all the 50 samples each of beef and buffalo meat. There were two bands, a thinner outer band and a thicker inner band against both the antigens. The bands were less distinct compared to the bands produced by antisera produced against antigen in distilled water. The hyper immune antibuffalo sera against meat extract in normal saline, when absorbed and tested in gel diffusion test, did not produce any precipitation bands against all the samples of buffalo meat and beef till the 6th day (Fig. 2).

c) Antibuffalo serum against meat extract in Freund's incomplete adjuvant

The antibuffalo serum raised with meat extract in Freund's incomplete adjuvant collected on the 15th day of immunization, when tested with buffalo meat and beef antigens, gave clear precipitation lines with both the antigens (in both dilution) within 18 hours. The band patterns were identical for both antigens with a thick

inner band and a thin outer band. This test was repeated with all the 50 meat samples each from buffalo and beef and results obtained were consistently similar. The absorbed serum when tested against all the samples of buffalo meat and beef, showed negative results with the absence of any visible precipitation lines when observed at 18 hours, 24 hours and 48 hours after charging (Fig. 3)

d) Antibuffalo serum against alum precipitated meat protein

50 samples each of beef and buffalo meat when tested by gel diffusion test, against the serum collected on the 15th day, gave identical precipitation lines after 18 hours of charging. The hyperimmune sera also, when tested with undiluted and diluted (1:10) antigens from all the samples of beef and buffalo meat produced precipitation lines. There were two thin precipitation lines with both beef and buffalo meat samples in all cases. The absorbed antiserum in double diffusion test against all the meat samples tested did not produce any precipitation reaction after 12 hours. On observations made at 18 hours, 24 hours, 48 hours and 6th day there was no observable reaction in any of the samples (Fig. 4).

e) Tests with antibeef serum to differentiate beef from buffalo meat

Antibeef serum against meat extract in distilled water

The antibeef serum raised against meat extract in distilled water when tested by doublediffusion in agar gel all the samples of beef and buffalo meat behaved in the same manner as in the case of antibuffalo serum.

The sera harvested on the 25th day of immunization gave precipitation lines, with beef and buffalo meat antigen after 18 hours. The bands were not so prominant and distinct compared to that produced with antibuffalo serum. The bands obtained against both beef and buffalo meat were similar without any distinguishable features.

The hyperimmune sera harvested at terminal bleeding also gave similar precipitation lines with undiluted and diluted (1:10) antigens within 18 hours, for both beef and buffalo meat.

When the heterologous antibodies in the antibeef sera were absorbed with freeze dried buffalo meat extract

and that antisera tested by gel diffusion with beef and buffalo meat samples, no lines of precipitation were produced until sixth day. The results were similar when the antiserum harvested on 25th day of immunization was absorbed and tested with beef and buffalo meat samples. The test results showed no visible change when observed at 18 hours, 24 hours, 48 hours and on 6th day of charging (Fig. 5).

f) Antibeef serum against meat extract in saline

Fifty samples each of beef and buffalo meat when tested with the antiserum collected on 21st day, gave positive precipitation reaction after 18 hours. When the test was performed using hyperimmune serum harvested on terminal bleeding, clear positive precipitation lines were observed in the case of both beef and buffalo meat antigen within 18 hours.

The absorbed antibeef sera against meat extract in normal saline gave no precipitation reaction in agar gel against homologous antigen samples and buffalo meat samples after 18 hours. There were no precipitation bands at 24 hours, 48 hours and on 6th day. The reactions

were similar when tested with absorbed sera of 25th day of immunization (Fig. 6).

Differentiation of buffalo meat from beef by immunoelectrophoresis

The results of immunoelectrophoresis with different antisera and antigens are given in chart 1.

a) Antibuffalo serum to meat extract in distilled water

This antiserum on electroimmunodiffusion gave three precipitin arcs with both homologous antigen and beef antigens. Similar precipitin arc patterns were observed with forty meat samples (20 beef and 20 buffalo meat) (Fig. 7). When the test was repeated with absorbed antiserum there was no line of precipitation in all the samples tested.

b) Antibuffalo serum against meat extract in saline

The immunoelectrophoresis of antibuffalo serum (against meat extract in saline) with buffalo meat and beef samples resulted in the production of three precipitin arcs in both cases. No changes in the number of precipitin arcs were noticed in both beef and

buffalo meat samples when the test was repeated (Fig. 8). The absorbed antisera on immunoelectrophoresis on agarose gel showed no reaction with all beef and buffalo meat samples.

c) Antibuffalo serum against buffalo meat extract in Freund's incomplete adjuvant

In immunoelectrophoresis with the antibuffalo serum (against Freund's incomplete adjuvant emulsified meat extract) all the samples of beef and buffalo meat reacted alike producing three identical precipitin arcs each (Fig. 9). When antisera was tested after absorption no precipitin arcs were produced against both buffalo meat and beef antigens in all the 20 tests.

d) Antibuffalo sera against alum precipitated meat protein

When this antisera was tested against beef and buffalo meat antigens by immunoelectrophoresis, all the meat samples gave three precipitin arcs. The absorbed antibuffalo serum to alum recipitated meat extract did not produce any precipitin arcs with both beef and buffalo meat antigens on immunoelectrophoresis.

Cross-reaction of mutton, chevon, pork and dog meat

a) Gel diffusion

When tested against antibuffalo serum (against meat extract in distilled water) all the 10 samples of chevon and mutton showed one precipitin band each while there was no precipitation with pork and dog meat antigens after 18 hours. When this test was repeated with antibeef serum (meat extract in distilled water) instead of antibuffalo meat serum, one precipitation line each for mutton and chevon and no line of precipitation for pork and dog meat were produced (Fig. 10 & 11).

b) Immunoelectrophoresis

The electrophoregrams of antibuffalo serum (against meat extract in distilled water) with chevon and mutton antigens showed oneprecipitin band in all cases but there was no precipitin band formation with pork and dog meat antigens (Fig. 12, 13, 14 & 15). The results were similar in all the cases. The results are summarised in chart 1. When the test was repeated using antibeef serum (against meat extract in distilled water) the results were the same as in the case of buffalo antiserum. The chevon

and mutton samples produced one precipitin arc each while there were no precipitin arcs for pork and dog meat samples.

Table 1. Showing the response of the rabbits to the various antigens used for raising antisera and the tire of the antisera produced

Type of the antigen	Interval between inoculations	Amount of antigen per inoculation	No. of animals showing precipitin in sera	Tire of antisera on gel diffusion test
Buffalo meat extract with Freund's incomplete adjuvant	weekly	(1+1) 2 ml	3/3	+++++
Alum precipitated buffalo meat extract	weekly	2 ml	2/2	+++
Buffalo meat extract in normal saline	4th day	5 ml	5/6	+++
Buffalo meat extract in distilled water	4th day	5 ml	5/5	++++
Beef extract in saline	4th day	5 ml	3/3	++++
Beef extract in distilled water	4th day	5 ml	4/4	+++

Table 2. Showing the minimum time required for the development of detectable level of antibodies in rabbits

Antigen	Immunisation schedule	Development of antibodies (days after initial injection)									
		7	9	11	13	15	17	19	21	23	25
Alum precipitated buffalo meat protein	2 injections	nil	nil	nil	nil	+	..	..	..	..	..
Buffalo meat extract in Freund's incomplete adjuvant	2 ,,	nil	bil	nil	nil	+	..	..	..	..	..
Buffalo meat extract in saline	4 ,,	nil	nil	nil	+	..	..	..	..	..	..
Buffalo meat extract in distilled water	3 ,,	nil	nil	+	..	..	..	..	..	..	..
Beef extract in saline	6 ,,	nil	nil	nil	nil	nil	nil	nil	+	..	..
Beef extract in distilled water	7 ,,	nil	nil	nil	nil	nil	nil	nil	nil	nil	+

Table 3. Showing compative efficacy of various antigens (Buffalo) to produce precipitin antibodies in rabbits as evidenced by the intensity of precipitin bands

Antigens	Hours after charging	Titre of antisera (intensity of bands) against			
		AL	SA	DW	FR
Undiluted buffalo antigens	12	+	++	++	++++
	18	+++	++++	+++++	++++++
Diluted buffalo antigens	12	+	+	++	++
	18	++	++	+++	++++
Undiluted beef antigens	12	nil	+	+	++
	18	+	++	+++	++++
Diluted beef antigens	12	nil	nil	+	++
	18	nil	+	++	+++

AL : Alum precipitated meat protein

SA : Meat extract in saline

D/W. : Meat extract in distilled water

FR. : Meat extract with Freund's incomplete adjuvant

Chart 1. Showing the immunoelectrophoretic patterns of different antigens and antisera

Antiserum	Antigens					
	BE	BU	G	S	P	D
Antibuffalo serum against:						
Alum precipitated meat extract	≡	≡	—	—	NCR	NCR
Meat extract in distilled water	≡	≡	—	—	NCR	NCR
Meat extract in normal saline	≡	≡	—	—	NCR	NCR
Meat extract with Freund's incomplete adjuvant	≡	≡	—	—	NCR	NCR
Antibeef serum against						
Meat extract in normal saline	≡	≡	—	—	NCR	NCR
Meat extract in distilled water	≡	≡	—	—	NCR	NCR

Key: BE : Beef, BU : Buffalo, G : Goat, S : Sheep, P : Pig, D : Dog.
NCR : No cross reaction, Number of lines indicate number of precipitin arcs.

DISCUSSION

DISCUSSION

Rabbits

According to Zwann (1963) rabbits are the most suited animals for the production of precipitin antibodies because they produce precipitins which can aggregate antigens very strongly over a wide antigen antibody ratio. The results of the present study also revealed that the rabbits are best suited for the production of precipitin antibodies, as all the rabbits except one, withstood the full course of immunization and produced adequate quantities of precipitin antibodies.

Rabbits which received beef antigens took a longer period for the development of detectable levels of antibodies compared to those received buffalo meat antigens. One of the rabbits from the group which received buffalo meat extract in saline failed to produce antibodies in their serum even after eight injections and such negative responses in rabbits to antigens were reported by Mohandas (1980).

Though Garvey *et al.* (1979) reported that the

Newzeland and Dutch strains of rabbits are best suited for antiserum production in the present study it was found that the local strains are good enough to produce antisera with satisfactory antibody titre for doing gel diffusion test.

Antigens and the route of administration

Though Singh and Yadav (1962) reported that intraperitoneal route of innoculation was not effective, in this study it was found to be very satisfactory as it produced high titre antiserum without the risk of anaphylaxis which can be expected when the protein antigens are administered intravenously.

In the present work all the four buffalo antigens and the two beef antigens were found to be potent to produce precipitins in the rabbit sera. Warnecke and Saffle (1968) have reported that the serum raised against meat extract in saline did not react with homologous or heterologous antigens in gel diffusion tests. But the results in the present study revealed that the meat extract in saline was an effective antigen to raise antisera which reacted with homologous antigens in the gel diffusion tests. Immunization schedule and the dosage

adopted by Warnecke and Saffle (1968) was different from the present study. The initial dosage of antigen given by them was 30 to 45 mg. Christian (1970) reported that the protein content of more than 10 mg in the initial injections failed to produce precipitins in rabbits. In the present experiment the initial dose of antigen contained only 0.9 mg protein.

Proom (1943) got high titred species specific precipitin sera from rabbits after a single injection of alum precipitated protein antigens within 20 days of immunization. According to Warneck and Saffle (1968) alum precipitated tissue extracts gave very little antibody reponse in gel diffusion tests. The antibodies so obtained were non-species-specific and after absorption failed to detect even antigens of homologous species. Katsube and Imaizumi (1968) reported that none of their rabbits that received alum precipitated raw beef protein produced antibodies. In the present study alum precipitated proteins produced a detectable level of antibody by the 15th day and after the 2nd injection. This is in agreement with the findings of Proom (1943) as far as the effectiveness of the antigen is concerned but do not

agree with the findings of Katsube and Imaizumi (1968). Warnecke and Saffle (1968) reported that the tissue proteins emulisified in Freund's adjuvant was the most potent antigen since it gave the strongest precipitin antisera. Herbert (1968) reported that in some cases a response to antigens given in Freund's adjuvant is about 500 times more than that of antigen given in saline In the present study also the meat extract in Freund's incomplete adjuvant produced antiserum with the highest titre.

Minimum time required for the development of detectable level of antibodies

It has been reported that the type and sequence of antibody production are associated with the nature of antigen and the routes of administration. Precipitins appear rapidly in the serum after antigens have been administered in saline (Orlans et al. 1961; Richter et al. 1962). In the present work the shortest time required for the appearance of detectable levels of antibodies was for meat extract in distilled water in case of buffalo meat antigen and meat extract in saline for beef. The variation in time interval was only one day and this may

be due to the individual variations among rabbits. It took almost the same length of time for the production of detectable levels of antibodies with saline as well as distilled water extracted meat antigens.

Gel diffusion test and cross reaction

The results of the gel diffusion tests conducted using the antisera raised against different types of buffalo meat and beef antigens indicated that these sera are not species specific, and reacted with both beef and buffalo meat alike. This shows that these antisera contained overlapping antibodies as indicated by Hafeze et al. (1961); Pinto (1961); Katzube and Imaizumi (1968) and Sherikar et al. (1979).

Singh and Yadav (1962) observed that there is a close species-relationship between cow and buffalo proteins. Pandey and Pathak (1966) reported that antisera raised in rabbits fail to distinguish cow and buffalo tissue proteins. Katzube and Imaizumi (1968) compared the antisera produced by different types of buffalo and bovine antigens and found that though antiserum raised against meat extract in Freund's incomplete adjuvant provided sera of highest titre, none of these antisera

were effective in differentiation of beef and buffalo meat samples. Proom (1943); Pinto (1961); Katsube and Imaizumi (1968) and Warnecke and Saffle (1968) reported that these cross reacting antibodies could be removed by absorption with heterologous antigens. Fireman et al. (1963) and Zwann (1963) used small quantities of freeze dried antigen for absorption of cross-reacting antibodies in order to retain the efficacy and to avoid undue dilution of sera. The methods suggested by the above workers were adopted in this study to absorb cross-reacting antibodies, but the results revealed that the efficacy of the antiserum was not retained after absorption.

Single injection of antigen was reported to produce species specific antibody (Proom, 1943) while Williams et al. (1964) noticed that the cross-reactivity and polyvalency usually increased with time after immunization. Singh and Yadav (1962) established close species relationship between cow and buffalo and Pandey and Pathak (1966) failed to distinguish cow and buffalo tissue proteins with antisera raised in rabbits. Omland (1963); Murasehi et al. (1965) and Rodky and Freeman (1970) reported that early bleedings generally provided high specificity

sera. In the present experiment the sera collected by early bleeding of the rabbits were not at all species-specific and was cross-reacting with the heterologous antigens. The trials to absorb out the cross-reacting antibodies were uniformly unsuccessful as reported by Proom (1943).

This finding is contrary to the reports of Tagore et al. (1977). They could make species-specific antisera against beef and buffalo meat by absorption of the hyperimmune sera with heterologous whole serum. The results of the gel-diffusion test also indicated that the dilution of test antigens will not reduce the cross reactivity of the antiserum.

Immunoelectrophoresis

Differentiation of beef and buffalo flesh by immunoelectrophoresis using various antisera was also unsuccessful. The findings revealed that cow and buffalo are closely related species and antiserum raised against the beef and buffalo meat antigens will contain cross-reacting antibodies, which overlap. Immunoelectrophoretic patterns produced against muscle extracts of bullock and buffalo with unabsorbed homologous and heterologous antisera were

indistinguishable. However, Ramadass and Misra (1981) reported that buffalo antisera gave two distinct precipitin arcs against its homologous antigens and there was no cross reaction with beef antigen. They also reported that beef antisera reacted to both beef and buffalo antigens with three and two arcs respectively. This type of reaction is rather difficult to occur as there is very close species relationship according to Singh and Yadav (1962) and Pandey and Pathak (1966).

In the present study when antibuffalo serum was tested against beef antigen and antibeef serum against buffalo meat antigen, three arcs of precipitation each were obtained in both cases. Mutton and chevon also cross reacted with these antisera with one precipitation arc each. Ramadass and Misra (1981) reported that there was no cross-reaction between antibullock sera with sheep and goat antigen and antibuffalo sera with sheep antigen.

The results of the study showed that strong antigenic relationship existed between buffalo and cattle and as such there is very strong cross-reaction between the antibody against one species and antigens of the other. When there is such close species-relationship

between these two, absorption of the antisera of one species with the antigen from the other would result in nondetectable levels of remaining antibodies. There would definitely be fractions of antibodies which should remain after absorption but it would be so little that it is not detectable by gel diffusion or immunoelectrophoresis.

SUMMARY

SUMMARY

Adulteration and substitution are common frauds encountered in meat industry in many parts of the world. Serological methods are considered as one of the reliable techniques for the detection of such fradulant practices. So this study, differentiation of buffalo meat from beef by serological methods was undertaken. Immune sera was raised in rabbits maintained in the laboratory against four antigen preparation from buffalo meat viz. meat extracts in distilled water and saline, alum precipitated meat protein and meat extract in Freund's incomplete adjuvant. Antibeef serum was raised against meat extract in saline and distilled water. The antigen preparation in saline and distilled water were administered intraperitoneally at four days intervals while the antigens with adjuvants were administered intramuscularly at weekly intervals. A total of 10, 10, 6 and 2 injections were administered in the case of antigen preparations in saline, distilled water, alum precipitated meat and meat extract in Freund's incomplete adjuvant respectively.

Though all the antigen preparation proved to be

potent in producing antisera, meat extract with Freund's incomplete adjuvant was found to be most effective in raising high titred antisera in rabbits.

In the test bleeding done to determine the minimum time required for the appearance of detectable level of precipitin antibodies, buffalo meat in distilled water produced antibodies on the 11th day followed by the same in normal saline on 13th day. Buffalo meat antigen with adjuvants produced antibodies from 15th day of immunization. Beef antigens took a minimum time of 21-25 days.

The unabsorbed immune sera against both buffalo meat antigens and beef antigens were found to contain cross-reacting antibodies against each other as evidenced by the similarity of the bands formed against both antigens, in gel diffusion and on immunoelectrophoresis. It was also observed that all these sera were loosing their precipitin activity to produce any visible reaction after absorption with heterologous antigens, in gel diffusion tests as well as in immunoelectrophoresis, when tested with homologous and heterologous antigens.

Fifty samples each of beef and buffalo meat were tested and all the samples gave uniformly similar results.

A minimum time of five hours was required for the development of precipitation bands in the gel diffusion tests using antibuffalo meat sera with beef and buffalo meat antigens. Both antibuffalo and anticow sera cross reacted with mutton and chevon by forming precipitin bands. However no cross-reactions were observed with pork and dog meat antigens in gel diffusion and immunoelectrophoresis.

The results of the study shows that serological tests like, gel diffusion and immunoelectrophoresis are not suited for the differentiation of buffalo meat from beef because of very close antigenic relationship between these two species. The absorption of antisera with heterologous antigen was uniformly unsuccessful, to produce species specific antisera, as the process, resulted in leaving only undetectable levels of antibodies in the absorbed antiserum.

REFERENCES

REFERENCES

REFERENCES

Abraham, J. (1982) Differentiation of meat from different species of animals by agarose isoelectric focussing. Kerala J. vet. Sci. 13 : 261-266.

*Aladjem, F. (1964) J. Immunol. 93 : 682-695. Cited by Crowle (1973).

*Aladjem, F.; Jaross, R.W.; Paldino, R.L. and Lackner, J.A. (1959) J. Immunol. 83 : 221-231. Cited by Crowle (1973).

Babiker, S.A.; Glover, P.A. and Lawrie, R.A. (1981) Improved Methodology for the electrophoretic determination of horse meat in heated food stuffs. Meat Science. 5 : 473-477.

Baker, F.J. and Breach, M.R. (1980) Medical Microbiological Techniques. Butterworth, London. pp. 396-397.

Brandley, P.J.; Migaki, G and Taylor, K.E. (1966) Meat Hygiene. 3rd edn. Lea and Febiger. Philadelphia. pp. 356-367.

Chase, M.W. (1967) Methods in Immunology and Immunochemistry. Vol. I. Edited by Case, W.M. and Williams, A.C. Academic Press . London. pp. 19.

*Christian, C.L. (1970) Immunology. 18 : 457-466. Cited by Crowle (1973).

*Croisille, Y.(1962) C.R. Soc. Biol. 156 : 1221-1225. Cited by Crowle (1973)

Cromwell, H.W. (1925) Quantitative relations between antigen and antibody in the precipitation reaction. J. Infectious diseases. 37 : 321-328.

Crowell, G.K. (1944) Detection of horse meat in admixtures with pork or beef. J. Assoc. Off. Agr. Chem. 27: 449-451.

Crowle, A.J. (1973) Immunodiffusion 2nd edn. Academic Press. New York. pp. 356, 367., 65-96,124, 276-302,305.

Dean, H.R. and Webb, R.A. (1926) Influence of optimal proportions of antigen and antibody in the serum in precipitation reaction. Journal of Pathology. 29 : 473-492.

*DePagondo, A.P. and Bordner, M. (1943). Preparation of high titre precipitating sera for the determination of the animal origin in meat products. Biol. Tech. Dive. Gen. Agric. Canad. Cited by Shanmugam and Ranganathan (1972).

*DeCarvalho, S. (1960). J. Lab. Clin. Med. 56 : 333-341. Cited by Crowle, (1973)

*Dilworth, T.G. and Mc Kenzie, J.A. (1970) Attempts to identify meat of game animals by starch gel electrophoresis. J. Wildl. Mgmt. 34 : 917-921. Vet. Bull. 41 (1971) Abstract No. 2680.

*Dodin, A. (1963) Ann. Inst. Pasteur. 105 : 1098-1107. Cited by Crowle, (1973)

*Durrand and Schneider (1962) Arch. Inst. Pasteur Tunis. 39 : 137-151. Cited by Crowle (1973).

*Edelmann, R., Mohler, J. and Eichhorn, A. (1943). Text Book of Meat hygiene. 8th edn. Cited by Shunmugam and Ranganathan (1972).

*Evans (1957) Manual of Microbiological Methods. Mc Graw - Hill Book Co. Inc. New York. pp. 201. Cited by Shunmugam and Ranganathan (1972).

Fugate, G. and Penn (1971) Immunodiffusion technique for identification of animal species. J. Assoc. Off. Agr. Chem. 54 : 562.

Furminger, I.G.S. (1964) Species specificity of bovine, pig, sheep and horse myosins. Nature. London. 202 : 1332-1333.

Garvey, J.S.; Cremer, N.E. and Susdorf, D.H. (1977) Methods in Immunology. 3rd edn. Benjamin -London pp. 1, 37, 71, 273, 313.

Ginsberg, A. (1948) The differentiation of meats by precipitation test. Vet. Rec. 60 : 683-685.

*Glenn, W.G. (1961) School of Aerospace Medicine, USAF - Rep. No. 62-4 pp. 1-7. Cited by Crowle (1973)

*Guptha, S.S. and Hilditch, T.P. (1951) Biochem. J. 48 : 19. Cited by Weinstock (1953).

*Hafeze, N.M.; Ezzat, N.M. and Saad, L. (1961) Precipitation test for identification of meat sources. J. Arab. vet. med. Assn. 21 : 459-466. (J. Sci. Fd. Agric. 4711. 1963)

Hayden, A.R. (1979) Immunological detection of ovine, porcine and equine flesh in beef products with antisera to species myoglobin. J. Fd. Sci. 44 : 494.

*Herbert, W.J. (1968) Immunology. 14 : 301-318. Cited by Crowle (1973)

Holbrow, E.J. and Reeves, W.G. (1977) Immunology in medicine. Academic Press, London. pp. 180.

*Holland, N.H.; Hong, R., Davis, N.C. and West, C.D. (1962) J. Pediatr. 61 : 181-195. Cited by Crowle (1973)

Hynds, C.E. (1951) Report on Horse meat in Ground meat. J. Off. Agr. Chem. 34 : 355-357.

*Ishikawa, J. (1960) Hokkaido. J. med. Sci. 35 : 237-257. Cited by Katsube and Imaizumi (1968).

*Jameson, J.E. (1969) J. Clin. Pathol. 22 : 515-518. Cited by Crowle (1973)

*Jacob, T. (1976) Food Adulteration. Mc Millan & Co. of India Ltd. New Delhi. pp. 2.

Kaiser, K.P.; Matheis, G.; Kamita-Durrmann, C. and Belitz, H.D. (1980a) Identification of animal species in meat, fish and derived products by means of protein differentiation with electrophoretic methods. Z. Lensmittel. Unters. Forsch. 170 : 334-342.

Kaiser, K.P.; Matheis, G.; Kamita-Durrmann, C. and Belitz, H.D. (1980 b) Identification of animal species in meat fish, and derived products by means of protein differentiation with isoelectric focussing in polyacrilamide gel. II Qualitative and quantitative analysis of raw binary meat mixtures by Isoelectric focussing. Z. Lensmittel. Unters. Forsch. 171 : 415.

Kamiyama, T., Katsube, Y. and Imaizumi, K. (1978 a) Serological identification of animal species of meat by passive hemagglutination inhibition test using cross-reacting antiserum albumin antiserum. Jap. J. Vet. Sci. 40 (6) 653-661.

Kamiyama, T., Katsube, Y. and Imaizumi, K. (1978 b) Serological identification of animal species of meat using species specific antiserum albumin antibodies obtained by immunoadsorbant chromatography. Jap. J. Vet. Sci. 40 (6) 663-667.

Kaplan, E. and Buck, T.C. (1951) Detection of horse meat by biological precipitation test. J. Milk. Fd. Technol. 14 : 66-67.

Katsube, Y. and Imaizumi, K. (1968) Serological identification of animal meats. Jap. J. Vet. Sci. 30 : 219-232.

Keay, G. and Doxy, D.L. (1982)a Species characteristics of serum proteins demonstrated by gel electrophoresis. Vet. Res. Commun. 5 : 263-270.

Keay, G. and Doxy, D.L. (1982 b) Species characteristics of serum proteins demonstracted by gel electrophoresis. A comparison of serum proteins- Electrophoretic pattern of young and adult animals. Vet. Res. Commun. 5 : 271-276.

*Krause, R. (1897) Wiener Klin Wochenschr. 32 : 736. Cited by Merchant and Packer (1967).

*Kubota, (1967) 63rd Ann. meeting. Jap. Soc. Vet. Sci. Cited by Katsube, Y. and Imaizumi, K. (1968).

Kwapinski, J.B.J. (1972) Methodology of Immunochemical and Immunological Research. Wily - Inter Science. pp. 238, 259, 260.

*Landsteiner, K. (1947) Specificity of serological reactions. Harvard University Press, Cambridge. pp. 18. Cited by Pandey and Pathak (1966).

*Laron, Z. and Assa, S. (1962) Nature. London. 194 : 491-492. Cited by Crowle (1973)

Libbey, J.A. (1975) Meat hygiene. 4th edn. Lea and Febiger - Philadelphia. pp. 301.

*Lichter, E.A. and Dray, S. (1964) J. Immunol. 92: 91-92. Cited by Crowle (1973)

Makonkawkeyoon, S. and Haque, R. (1970) Annal Biochem. 36 : 422. Cited by Ramadass and Misra (1981).

Mattey, M.; Parson, A.L. and Lawrie, R.A. (1970) Quantitative identification of meat species after heating. J. Food Technol. 5 : 41-46.

*Maurer, P.H.; Gerulat, B.F. and Pinchuck, P. (1963) J. Immunol. 90 : 381-392. Cited by Crowle (1973).

Mendes, B. and Ribeiro (1971) Immunodiffusion as a method for meat differentiation. Arq. Esc. Vet. 23 : 263-267.

Merchant, I.A. and Packer, R.A. (1967) Veterinary Bacteriology and Virology. 7th edn. Iowa State University Press Iowa. pp. 157, 159 & 161.

Mohandas, N. (1980) Differentiation of beef from chevon by serological methods. M.V.Sc. Thesis submitted to the Kerala Agricultural University.

*Orlans, E., Rose, M.E. and Marrack, J.R. (1961) Immunology. 4 : 262-267. Cited by Crowle (1973).

Oswald, E.J. (1952) Serological methods in regulatory control of foods. J. Assn. Offi. Agri. Chem. 36 : 107-111.

*Ouchterloney, O. (1949) Antigen antibody reactions in gels. Acta. Path. Microbiol. Scand. 26 : 507. Cited by Garvey et al. (1977)

Pandey, R. and Pathak, R.C. (1965) Indian J. exp. Biol. 3 : 244.

Pandey, R. and Pathak, R.C. (1966) Agar diffusion studies on the specificity of cow and buffalo bones. Indian J. exp. Biol. 4 : 20-22.

*Penso, G. (1955) Seroprotein in the diagnosis of species in someprepared meats. Ann. inst. 7 : 231-254 (Food Sci. Abstr. 28 : 511-1955)

Pinto, F.C. (1961) Serological identification of ox, buffalo, goat and deer flesh. Brit. Vet. J. 117 : 540-544.

Prasad, V.S.S. and Misra, D.S. (1978) Differentiation of raw meats of different species of animals by using rivanol precipitated antisera. Indian J. Anim. Res. 12 : 5-7.

Prasad, V.S.S. and Misra, D.S. (1981) Differentiation of meats of different animals by muscle esterase pattern in different age and sex groups. Indian J. Anim. Sci. 51 : 211-214.

Proom (1943) Preparation of precipitating sera for the identification of animal species. J. Path. Bact. 55 : 419-426.

Ramadass P. and Misra, D.S. (1974) Differentiation of of meats of different species of animals by polyacrylamide-gel electrophoresis. Indian J. Anim. Sci. 44 : 844-847.

Ramadass, P. and Misra, D.S. (1981) Immunoelectrophoretic identification of meats of bullocks, buffalo, goats sheep, pig and chicken. Indian vet. J. 58 : 978-983.

*Richter, M.; Blumer, H.; Cua-Lim, F. and Rose, B. (1962) Can. J. Biochem. Physiol. 36 : 1105-1113. Cited by Crowle (1973).

*Scheidegger, J.J. (1955) Une micro-medthode de e immunoeletrophorese. Int. Arch. Allergy Appl. Immunol. 7 : 103. Cited by Crowle (1973)

Shanmugam, A.M. and Ranganathan, M. (1972) Differentiation of mutton and beef by precipitation test. Indian vet. J. 49 : 1024-1028.

Sherikar, A.T.; Ajinkya, S.M.; Khot, J.B. and Vaidya, A.M. (1979) Differentiation of meats by gel diffusion techniques. Indian J. Anim. Sci. 49 : 350-356.

*Sinel, H.J. and Mentz, I. (1969) Berl-Munch. tierarztl. Wschr. 82 : 85. Cited by Ramadass and Misra (1981).

Singh, B.S. and Yadav, P.C. (1962) Close species relationship demonstrated by the precipitation test between cow and buffalo. Indian vet. J. 39 : 40-42.

Tagore, S.S.R.; Venkitesan, E.S. and Ramamoorthy, R. (1977) The differentiation of beef from buffalo flesh by immuno double diffusion test. Cheiron (Tamilnadu J. of Veterinary Science and Animal Husbandry) 6 : 134.

Thornton, H. and Gracey, J.F. (1974) Text Book of Meat Hygiene. 6th edn. ELBS and Tindal. London. pp.118-119.

*Uhlenhuth, P. (1901) Deutsche med. wschr. 26: 734. Cited by Oswald (1953).

*Uhlenhuth, P. (1905) Dtsch. med. wschr, 31: 1673. Cited by Weitz (1952).

*Van den Heever, L.W. (1962) Serological identification of meat from different species by the agar gel diffusion method. S. Afr. Vet. med. Assn. 33 : 215-221. (Vet. Bull. 32. Abst. No.4401 (1962)

Warnecke, M.O. and Saffle, R.L. (1968) Serological identification of animal proteins 1. mode of injection and protein extract for antibody production. J. Food Sci. 31 : 131-135.

Weinstock, A. (1953) A survey of the detection of horse meat by serological precipitation test. J. Milk Fd. Technol. 16 : 257-259.

Weitz, P. (1952) The antigenicity of serum of man and animals in relation to preparation of specific precipitating serum. J. Hyg. 5 : 275-294.

*Williams, C.A.; Hargis, G.K.; Sidwell, C.G. and Yakulis, V.J. (1964) Proc. Soc. Exp. Biol. Med. 115 : 61-64 (Crowle, 1973).

Willams, C.A. and Chase, M.W. (1967) Methods in Immunology and Immunochemistry. vol. I. Academic Press, New York. pp. 178-197.

Williams, C.A. and Chase, M.W. (1971) Methods in Immunology and Immunochemistry. vol. I. Academic Press, New York.

*William and Garber (1955) Immunoelectrophoretic studies on serum proteins. J. Immunol. 74 : 158 & 397. Cited by Garvey et al.(1977).

*Wilson, M.W. and Pringle, B.H. (1954) J. Immunol. 73 : 232-243. Cited by Crowle (1973).

*Zwann, J. (1963) Immunochemical Analysis of the Eye Lens during Development. Rototype, Amsterdam. Cited by Crowle (1973).

Zewigh, G. and Whitaker, J.R. (1967) Paper chromatography and Electrophoresis. Academic Press. New York. Vol. I. pp. 111-117.

* Reference not consulted in original.

Fig. 1

Set I. Central well - antibuffalo meat serum against meat extract in distilled water.

Wells 1 & 2 : buffalo meat antigens

Wells 5 & 6 : beef antigens

Set II. Central well - absorbed antibuffalo serum against meat extract in distilled water.

Wells 1 & 2 : buffalo meat antigens

Wells 5 & 6 : beef antigens

Fig. 2

Set I. Central well - antibuffalo meat serum against meat extract in saline

Wells 1 & 2 : buffalo meat antigens

Wells 5 & 6 : beef antigens

Set II. Central well - absorbed antibuffalo serum against meat extract in saline

Wells 1 & 2 : buffalo meat antigens

Wells 5 & 6 : beef antigens

Fig.3

Set I. Central well - antibuffalo meat serum against meat extract with Freund's incomplete adjuvant.

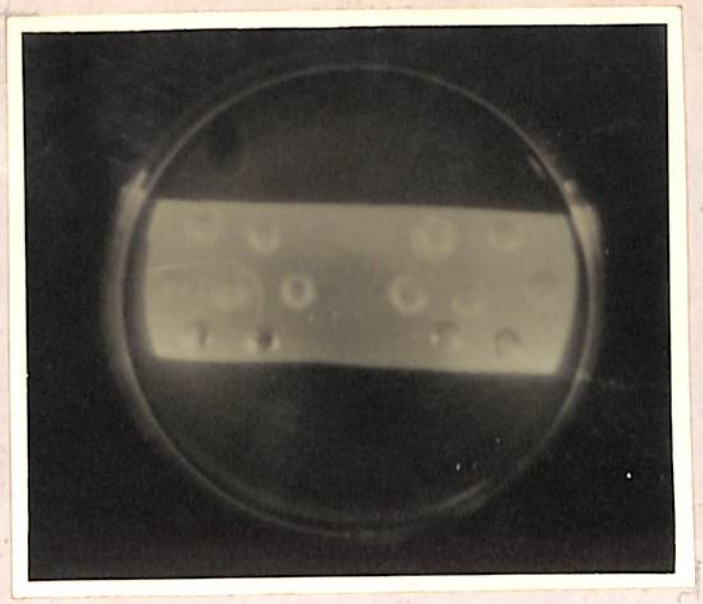
Wells 1 & 2 : buffalo meat antigens

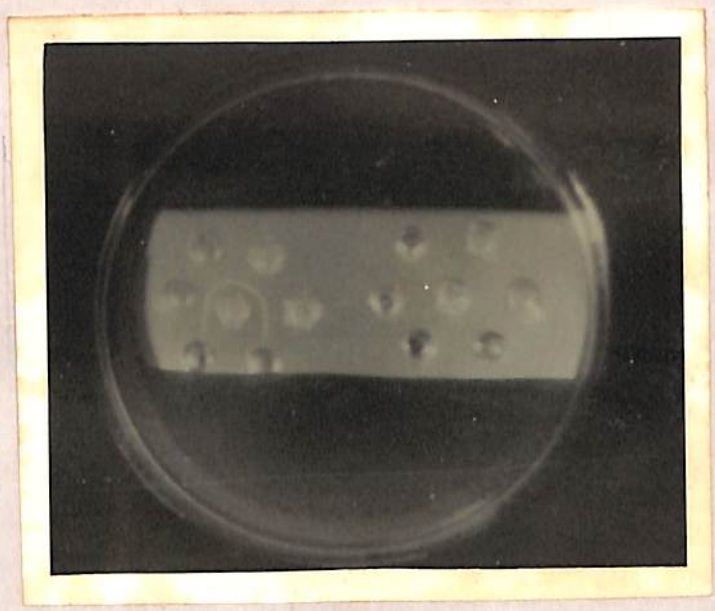
Wells 5 & 6 : beef antigens

Set II. Central well - absorbed antibuffalo meat serum against meat extract in Freund's incomplete adjuvant

Wells 1 & 2 : buffalo meat antigens

Wells 5 & 6 : beef antigens





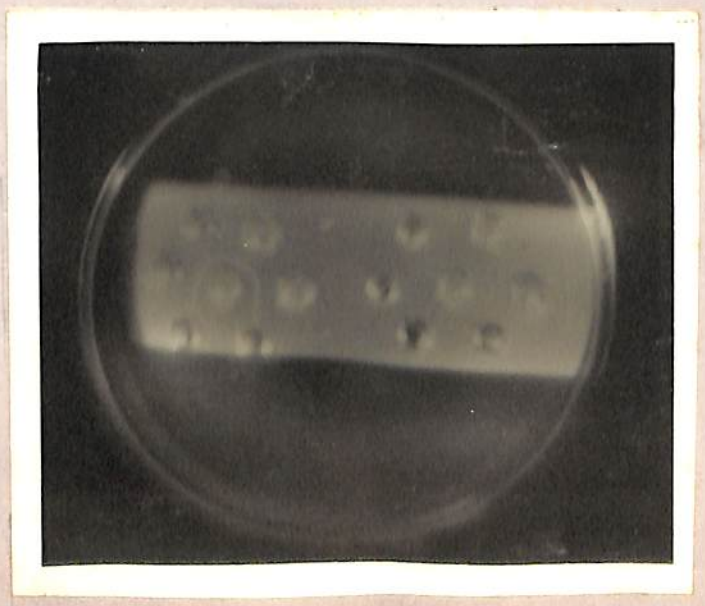


Fig. 4

Set I. Central well - antibuffalo meat serum against alum precipitated meat proteins.

Wells 1 & 2 : buffalo meat antigen

Wells 5 & 6 : beef antigen

Set II. Central well - absorbed antibuffalo meat serum against alum precipitated meat proteins

Wells 1 & 2 : buffalo meat antigen

Wells 5 & 6 : beef antigen

Fig. 5

Set I. Central well - antibeef serum against meat extract in distilled water

Wells 1 & 2 : buffalo meat antigen

Wells 5 & 6 : beef antigen

Set II. Central well - absorbed antibeef serum against meat extract in distilled water

Wells 1 & 2 : buffalo meat antigen

Wells 5 & 6 : beef antigen

Fig. 6

Set I. Central well - antibeef serum against meat extract in saline

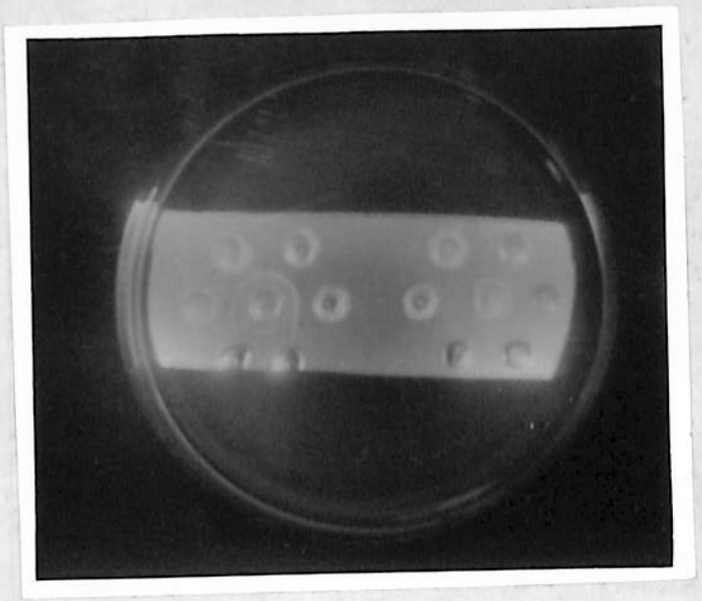
Wells 1 & 2 : buffalo meat antigen

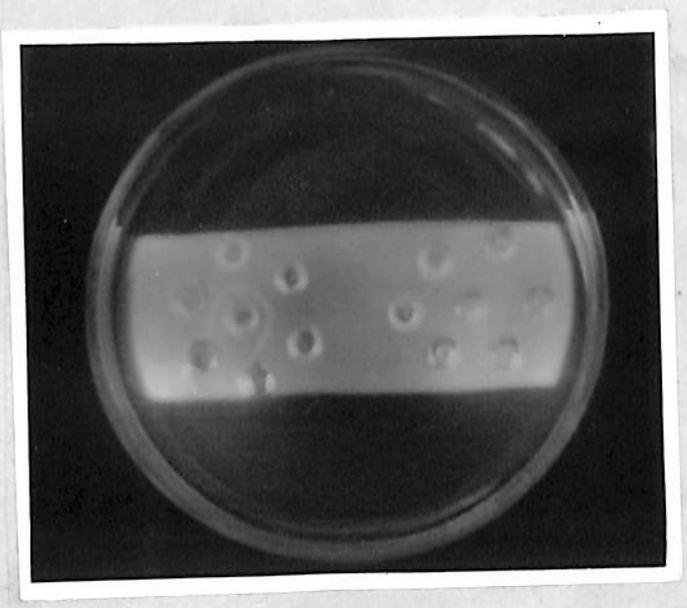
Wells 5 & 6 : beef antigen

Set II. Central well - absorbed antibeef serum against meat extract in saline

Wells 1 & 2 : buffalo meat antigen

Wells 5 & 6 : beef antigen





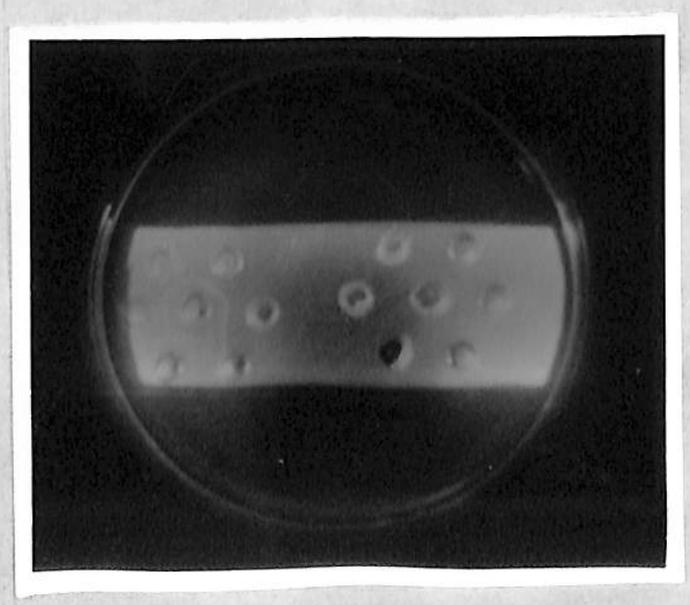


Fig. 7 : Central trough - antibuffalo meat serum against meat extract in distilled water

Upper well : beef antigen

Lower well : buffalo meat antigen

Fig. 8 : Central trough - antibuffalo meat serum against meat extract in saline

Upper well : beef antigen

Lower well : buffalo meat antigen

Fig. 9 : Central trough - antibuffalo serum against meat extract in Freund's incomplete adjuvant

Upper well : beef antigen

Lower well : buffalo meat antigen

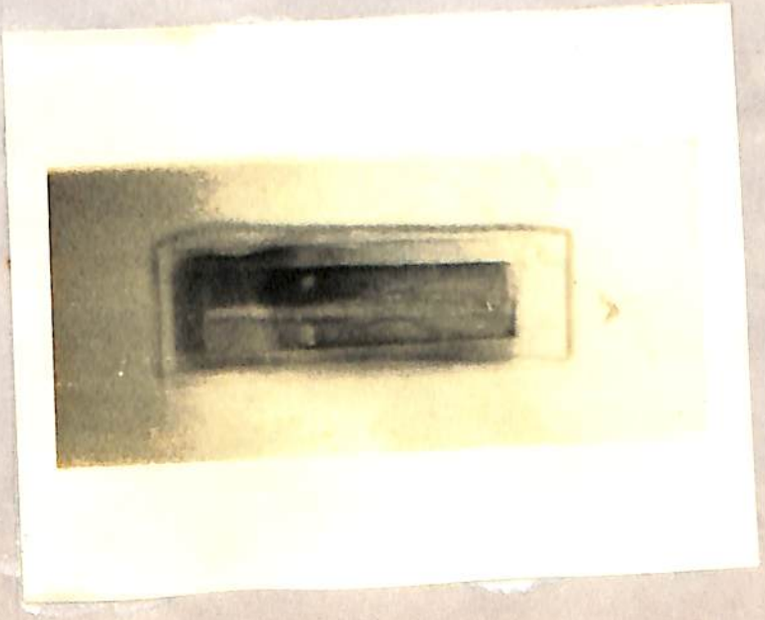


Fig. 10

Set I. Central well - antibuffalo meat serum against meat extract in distilled water

Wells 1 & 2 : buffalo meat antigen

Wells 5 & 6 : beef antigen

Set II. Central well - antibuffalo meat serum against meat extract in distilled water

Wells 1 & 2 ; mutton antigen

Wells 5 & 6 : chevon antigens

Fig. 11

Set I. Central well - antibuffalo meat serum against meat extract in distilled water

Wells 1 & 2 : buffalo meat antigen

Wells 5 & 6 : beef antigen

Set II Central well - antibuffalo meat serum against meat extract in distilled water

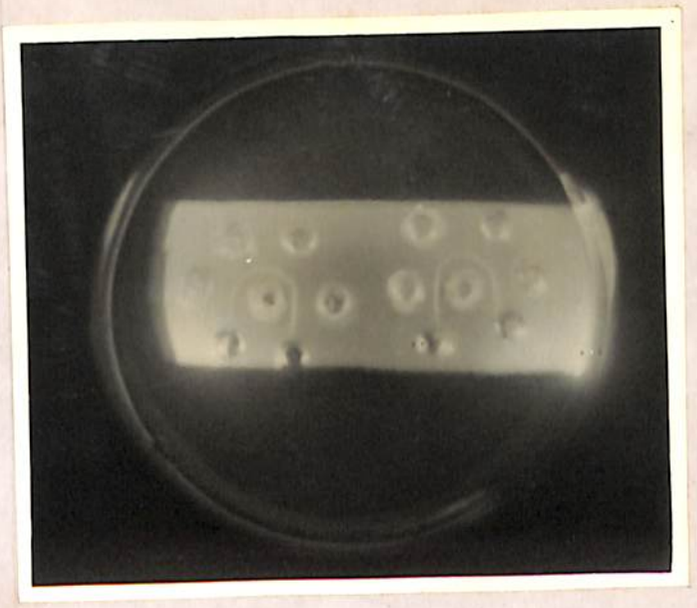
Wells 1 & 2 : dog meat antigen

Wells 5 & 6 : pork antigen

Fig. 12 Central trough - antibuffalo meat serum against meat extract in distilled water

Upper well : Chevon antigen

Lower well : buffalo meat antigen



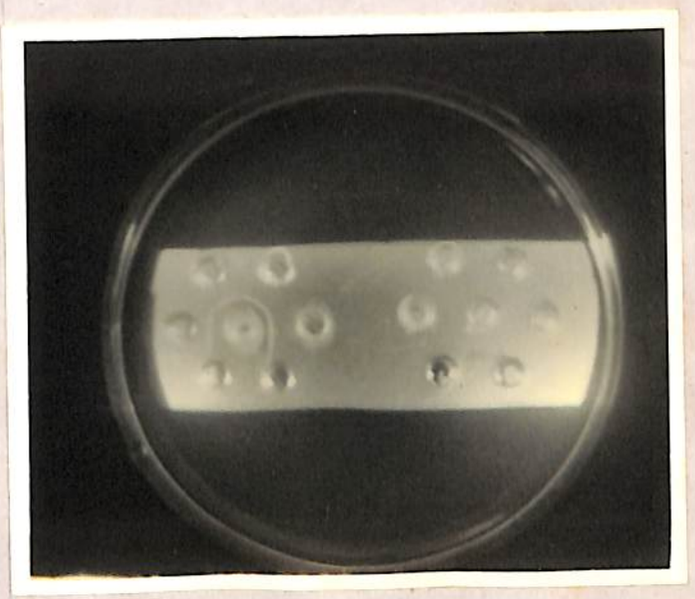


Fig. 13 Central trough - antibuffalo meat serum against meat extract in distilled water

Upper well : mutton antigen

Lower well : buffalo meat antigen

Fig. 14 Central trough - antibuffalo meat serum against meat extract in distilled water

Upper well : pork antigen

Lower well : buffalo meat antigen

Fig. 15 Central trough - antibuffalo meat serum against meat extract in distilled water

Upper well : Dog meat antigen

Lower well : pork antigen

DIFFERENTIATION OF BUFFALO FLESH FROM BEEF BY SEROLOGICAL METHODS

By

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ABSTRACT OF A THESIS

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ABSTRACT

Immuno-double-diffusion in agar gel and immuno-electrophoresis on agarose were concurrently done to differentiate buffalo flesh from beef using unabsorbed antibuffalo serum and the test antigens prepared from 50 samples each of buffalo meat and beef in saline. The tests were repeated with unabsorbed antibeef serum. Both unabsorbed antibuffalo meat serum and antibeef serum reacted similarly with beef and buffalo meat antigens producing identical precipitation bands. Three precipitin arcs each were produced by whole antibuffalo meat and antibeef sera with beef and buffalo meat test antigens in immunoelectrophoresis. When the antibeef and antibuffalo meat sera were absorbed with freeze dried heterologous antigens and tested by agar gel diffusion and by immunoelectrophoresis, both the sera failed to produce any visible reaction with buffalo meat and beef antigens prepared from test samples. Mutton and chevon cross reacted with antibeef and antibuffalo meat sera while pork and dog meat showed no cross-reaction.

Among the various antigens used for raising antisera in rabbits meat extract in Freund's incomplete adjuvant was found to be most potent. A minimum of 11 days was

required for the development of appreciable level of antibodies in rabbit serum with buffalo meat antigen in distilled water.

The time lapse for the appearance of visible precipitation lines, in gel diffusion tests was five hours with antibuffalo sera against buffalo meat and beef antigens.