Serological Characterization of Meat, Skin and Hair of Cow, Buffalo and Camel

गाय, भैंस व ऊंट के आमिष, चर्म व बालों का लासिकीय अभिज्ञान

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B. V. Sc. & A. H.

THESIS

Master of Veterinary Science

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Department of Medicine
College of Veterinary and Animal Science
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CERTIFICATE I

Dated : 17.5., 1994

This is to certify that Mr. Shubhendu Dixit has successfully completed the COMPREHENSIVE EXAMINATION held on 29th May, 1993, as required under the regulation for the degree of MASTER OF VETERINARY SCIENCE.

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This is to certify that this Thesis entitled "SEROLOGICAL CHARACTERIZATION OF MEAT, SKIN AND HAIR OF COW, BUFFALO AND CAMEL" submitted for the degree of MASTER OF VETERINARY SCIENCE in the subject of VETERINARY PUBLIC HEALTH of the RAJASTHAN AGRICULTURAL UNIVERSITY, BIKANER embodies bonafide research work carried out by Mr. Shubhendu Dixit, B.V.Sc. & A.H. under my guidance and supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during the course of investigation have been fully acknowledged. The draft of the thesis was also approved by the Advisory Committee on 29.4.94.

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This is to certify that this Thesis entitled "SEROLOGICAL CHARACTERIZATION OF MEAT, SKIN AND HAIR OF COW, BUFFALO AND CAMEL" submitted by Mr. Shubhendu Dixit, B.V.Sc. & A.H. to the RAJASTHAN AGRICULTURAL UNIVERSITY, BIKANER in partial fulfillment of the requirements for the degree of MASTER OF VETERINARY SCIENCE in the subject of VETERINARY PUBLIC HEALTH was after recommendation by the external examiner was defended by the candidate before the following members of the examination committee. The performance of the candidate in the oral examination on his thesis has been found satisfactory, we therefore, recommend that the thesis be approved.

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INTRODUCTION

1. INTRODUCTION

Meat has formed an important constituent of human diet since ages. It is highly nutritious, palatable and a rich source of proteins, vitamins and minerals. Animal proteins have a higher biological value as compaired to vegetables proteins. Hence, meat forms a rich wholesome diet.

Meat consumption practices vary from nation to nation and culture to culture with regards to the species to be used and the form in which it is consumed i.e. cooked, raw, frozen or lately as processed in the form of various products.

The meat production potential of India is immense, but the meat industry is still in its formative stages. This industry is largely handled by a few traditional and poor sections of the society. Though, lately several business houses as well as government enterprises have taken up meat processing and product marketing for indigenous consumption as well as export. These modern processing plants are confined to metropolis and can meet the demand of only a small percentage of meat consumers. As such the common man still depends on the traditional system.

Today, meat consumption in India is on increase due to shedding of age old social stigmas and growing awareness towards wholesome nutrition. It is becoming popular even in those communities which generally abstain from it. Thus, by observing the present trend, it can be safely inferred that the meat industry in India is on rise. But unfortunately, falsification of meat is also on the rising trend.

Twenty five to thirty per cent of edible meat sold in various parts of India is adulterated (Jacob, 1976). In India, apart from sheep and goat, other species of animals are also used in the meat industry for human consumption. The ban on slaughter is not applicable on buffaloes and therefore, religious or social ban on their slaughter does not come into picture. Buffaloes of any age can be freely and legally be slaughtered in any part of the country. Buffaloes contribute about 14 per cent to total meat production in the country (Sengar et al., 1988). Buffalo meat is cheaper in comparison to the meat of sheep and goat. This fact has deviated the poorer section of the society to consume buffalo meat. In various meat processing industries, buffalo meat is now regularly used for making various edible products.

In India, slaughter of cow is banned from religious point of view. Illegal slaughter of this animal should be seriously checked. Also, slaughter of donkey, horse and camel for edible use is not permitted by the authorities. Buffalo meat can be substituted by meat of donkey, camel, horse, cow and bullock. Such type of meat originate from dead animals and also by slaughter of unproductive animals.

There are certain well defined motives to substitute another gradient of lesser cost by some irresponsible people i.e. to earn more money or to meet out the preferential demand for a particular type of meat. Strict measures may check the marketing of poor quality meat and slaughtering of animals whose slaughter is banned in India. "The Food Adulteration

(Amendment) Act of India (1976)" declares food adulteration as an offence. But the detection of adulteration is cumbersome. It is difficult to identify the species on account of missing of major anatomical features since the people engaged in this malpractice, employ various methods to escape detection e.g. adulteration of meat of resembling species or homogenously mixing of meat in products.

Falsification of meat today is an international phenomenon and is prevalent widely. Hence, determination of origin of meat component(s) of meat products is an important and challenging task from the religious, consumer's satisfaction, human health, quality control and economic view points.

Apart from meat, skin and hair also help in identification of species. Pieces of meat, skin or hide and lumps of hair and wool of animals are confiscated from site by police or forest authorities (whose slaughter is banned in India) which pose difficulty in the exact identification of species to which they belong. Sometimes hairs are also found adhered to meat samples. Thus, identification of meat, skin or hide and hair is an important aspect in forensic science. The responsibilities of a veterinarian in checking fraudulent substitution and illegal slaughter is well understood. However, since visual grading and examination do not warrant complete safety, adulteration and illegal slaughter may go unnoticed in many cases.

The fraudulent adulteration of good quality meat with cheaper one is objectionable and ingestion of products containing undeclared meat may induce allergic reactions in sensitive individuals and also hurt religious sentiments of various communities like Hindu, Christian, and Jew, who have various prejudices against various kinds of meat. On account of this, consumer should be confident that the product received is the product paid for.

Anatomical, chemical and biochemical methods employed for identification of animal species are time consuming and inconclusive. Except on meat, literature is not ardently available in reference to identification of skin and hair but the identification through immunochemistry have proved to be of greater value for checking adulteration and identification of species of animals (Cook, 1963; Cook and Sturgeon, 1966; Thompson, 1961,1968; Simonsen, 1976). Antigenic specificity of protein molecules has been of further value in identification of animal species because of the fact that multiple molecular forms of protein provide a naturally built in genetic marker which help in determining variability in different species. Since the inherited protein variation has already been studied, thus identification of an adulterated and controversial sample is easily possible through these eastablished processes.

Considering all these facts, it was planned to attempt to find out the antigenic determinants of meat, skin and

hair proteins of the animal species under study and to differentiate them by serological techniques using antispecies serum. Antisera required for these tests was produced in phylogentically different animal i.e. rabbit.

Thus, an attempt was made for identification of different species by using sero-immunological techniques applied to meat, skin and hair as this can contribute a valuable aid in checking malpractices. The study was conducted with the following objectives : -

(1) To characterize the meat antigens of cow, buffalo and camel by using gel diffusion test.

(2) To characterize the skin antigens of cow, buffalo and camel by using gel diffusion test.

(3) Antigenic characterization of hairs of cow, buffalo and camel by using gel diffusion test.

(4) To study the agar gel electrophoretic pattern and immunoelectrophoretic characterization of meat, skin and hair antigens of cow, buffalo and camel.

(5) To study the correlation among meat, skin and hair antigens of cow, buffalo and camel. Absorption technique will be followed to overcome the presence of cross reactions if existing among the meat, skin and hair antigens of the species under study.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Some published reports are available regarding the serological differentiation of meat of various species. Hardly few appear to have been undertaken to differentiate skin and hair serologically as it is seen from paucity of available literature.

Identification of intact carcasses poses no difficulty due to their anatomical and morphological characters. Sometimes difficulties are faced in identification, as carcasses are dressed prior to submission or only skin and lumps of hair of animals are confiscated by the forest authorities or police in vetero-legal cases. Keeping in view these difficulties, certain methods have been evolved depending upon types of antibodies and their reaction to homologous antigens. The animal protein depict a high degree of individuality which is reflected in various serological method. These methods are so versatile that they are being commonly used for confirmation of different species in laboratories.

Species identification of animals have been performed by immunological methods such as "Tube precipitation test" (Ginsberg, 1948; Weitz, 1952; Pandey and Pathak, 1975), "Double gel immunodiffusion test" (Fugate and Penn, 1971; Sherikar et al. 1979; Tao and Poumeyrol, 1989), "Haem-agglutination test" (Kwapinski, 1965), "Counter immunoelectrophoresis" (Sinell and Mentz, 1969; Ramadass and

Misra 1981; Raut et al. 1988), "Starch gel electrophoresis" (Ramadass, 1972), "Disc electrophoresis", (Matoltsy and Matoltsy, 1963, Shechter et al. 1969, Simonsen, 1971) and "Immunoelectrophoresis", (Simonsen, 1976).

2.1. Immunizing Antigens

DeFagonde and Bodner (1943), Kaplan and Buck (1951) and Shaw et al. (1983) recommended the use of serum as immunizing antigens for raising anti-species serum in rabbits.

Proom (1943), Weitz (1952), Weitz and Glasgow (1956), Heever (1962), Soetarjo (1964) and Pandey and Pathak (1966) recommended alum-precipitated serum as immunizing antigen.

Oswald (1953), Warnecke and Saffle (1968), Munday et al. (1974) and Sherikar et al. (1979) advocated the use of saline extract of muscle as immunizing antigen.

Ramadass and Misra (1981) used alum-precipitated muscle extract for raising anti-species serum in rabbits.

Sinell and Mentz (1969) and Heever and Marais (1975) used the heat denatured muscles extracted with urea as immunizing antigen for raising antisera in rabbits.

Kamiyama (1976) and Kamiyama et al. (1978) used commercially available serum albumins for raising antisera in rabbits.

Herran and Meliton (1961) and Bubloz (1962) used both serum and muscles extract as immunizing antigens.

Kirrane and Robertson (1968) used Neutral-salt-soluble collagen (NSC), Tyrosylated neutral-salt-soluble collagen (TNSC), denatured NSC and TNSC and collagenase digested NSC and TNSC as immunizing antigens for raising antisera in rabbits and guinea pigs against rat collagen.

Purohit *et al*. (1982) used wool lysate for raising anti species serum in rabbits against sheep wool. They dissolved the wool sample in 10 per cent sodium hydroxide and subsequently dialysed, lyphollised, centrifuged and finally concentrated it by freeze drying.

Lahiri *et al*. (1983) successfully used the technique of freezing and thawing for preparation of skin extract of a fish (*Heteropneustes fossilis*).

Purohit *et al*. (1987) used freezing and thawing technique for preparation of cell membrane and flagellar antigens of *Trypanosoma evansi*.

Jain (1993) used the saline extract of meat of avian species as immunizing antigen and used the technique of freezing and thawing for preparing the antigens.

2.2. Immunization

Ginsberg (1948) produced specific antiserum for horse meat in rabbits by intra-peritoneal route.

Evans (1957) suggested that rabbit is the best laboratory animal for superior antibody production.

Boyd (1966) found that the antisera produced in rabbits against antigens, differentiated minor antigens amongst distantly placed animal species.

Hecht *et al*. (1943) raised specific anti-skin antibody by intra-muscular injection of homologous antigen and intra-dermal injection of staphylococcus toxoid. They reported that homologous skin antigen when used alone resulted in little if any, antibody formation.

Purohit *et al*. (1982) raised antisera in rabbits against sheep wool.

2.3. Inoculation Schedule

Fireman *et al*. (1963) reported that most of the animal species responded slowly on initial stimulation with an antigen, but much more rapidly on later stimulations. Sheep produced more precipitins when antigen was injected initially with incomplete Freund's adjuvant.

Campbell *et al*. (1970) applied a schedule of deep intra-muscular injection of meat antigen emulsified with Freund's complete adjuvant at weekly interval for five weeks and test bleeding was performed after seven days of the last injection.

Kwapinski (1972) recommended seven injections of meat antigens at four days interval and harvesting the serum after four days of the last injection. The fourth and fifth injection were given with Freund's incomplete adjuvant.

Shunmugam and Ranganathan (1972) applied a schedule of three intra-peritoneal inoculations of meat antigens per week on alternate days and harvested the antiserum after fifteen days of the last inoculation.

Kamiyama (1976) applied a schedule of five inoculations of meat antigen with an interval of one week between each inoculation and harvested the serum after one week of the last inoculation.

Bakshi (1981) raised antisera in sheep (rams and ewes both) by giving an initial course of seven injections of saline extract of meat with Freund's incomplete adjuvant at four days interval, which did not stimulate any demonstrable antibodies. However, a second course of three injections, each of 5ml pooled serum with 1ml Freund's incomplete adjuvant, given four days apart and fourth injection of 10ml inactivated serum with 2ml adjuvant produced a satisfactory antibody response.

Ramadass and Misra (1981) raised antisera in rabbits against meat extract by giving four intra-muscular injections on alternate days, rested for ten days and than two subsequent injection in similar manner and harvested the serum after fifteen days of the last injection.

Jain (1993) raised antisera against muscle extracts of various avian species by giving subcutaneous injection of meat antigen along with Freund's incomplete adjuvant on 0, 3rd, 6th and 16th day and harvested the antiserum after four days of the last inoculation.

Kirrane and Robertson (1968) raised antiserum against rat skin (collagen) by giving first injection in rabbits into the foot pad, second intra-dermal injection in between the scapulae after two weeks of the first injection and then at fortnightly interval for six months intravenously. Blood for collection of antiserum was taken prior to each injection. They also raised antisera against the same in guinea pigs.

Purohit et al. (1982) raised antiserum in rabbits against sheep wool by giving three injection on alternate days and fourth injection on fifteenth day and serum was harvested on twentieth day.

2.4. Double Gel Immunodiffusion Test

The species-specific character of proteins was used in serological tests to differentiate meat, skin and hair of different species.

Bechold (1905) was first to describe immunodiffusion test in gels.

Oudin (1949) reported precipitation in transparent gel by allowing the reagents to diffuse towards each other and he further used this to analyse heterogenous antigen-antibody systems.

Ouchterlony (1949, 1953, 1968) differentiated three principal types of reactions when related antigens in adjacent wells were allowed to react with antibodies against various antigenic determinants diffusing from the central well.

Mansi (1958) modified Ouchterlony's technique so as to use extremely small quantities of the reagents with minimum time of appearance of precipitin bands.

Yakulis and Heller (1959) recommended the reapplication of melted agar into the bottom of wells before receiving the reagents. They also reported that the agar slides could be stored in a moist chamber for some days.

Application

Pike and Sulkin (1957) were amongst the first to employ the agar gel diffusion test for the detection of adulteration of beef with horse meat.

Aladjem et al. (1959) reported the double immunodiffusion test to be more sensitive than the single diffusion test.

Schlotfeldt and Simon (1960) applied Ouchterlony's method of double diffusion in agar gel for the detection of different kinds of meat in crude sausages.

Pinto (1961) reported significant cross-reactivity of unabsorbed sera of rabbit origin amongst proteins of ruminants.

Bubloz (1962) applied double diffusion gel method for the identification of meat extract and detected the principal horse meat antigen in a mixture of one part of horse meat mixed in nine parts of beef.

Hay (1962) identified the origin of animal protein by agar gel diffusion method and reported the degree of precipitation to be correlated to the zoological family of the animal.

Heever (1962) identified the meat from sheep, cattle and horses by using agar gel diffusion test and further stated the successful application of the technique to Bilton (air dried salted strips of meat).

Singh and Yadav (1962) demonstrated close relationship between cow and buffalo by using the precipitation test with rabbit antisera.

Soetarjo (1964) reported that anti-horse, anti-pig and anti-dog sera prepared by Proom's (1943) method showed high specificity in gel diffusion tests, only forming precipitin lines between beef, mutton, buffalo and goat meat and their antisera. He further stated that, sun-dried meat and meat cooked at $90^{o}C$ for 20 minutes gave precipitin lines with homologous antisera.

Pandey and Pathak (1966) compared the antisera raised in rabbits and in buffalo by cross immunization with alum-

precipitated bone and meat extracts reacted specifically forming three precipitin lines with each homologous antisera raised in rabbits and cross-reacted within the species of bones and meat extracts.

Warnecke and Saffle (1968) observed that anti-beef serum reacted with beef serum and beef extract forming two definite precipitation bands. They recorded one precipitin band between beef antiserum and lamb muscle extract. After absorption of anti-beef serum with lamb, horse and pork freeze dried meat extracts, they observed that it becomes species specific and reacted only with beef extract and beef serum.

Anon (1970) reported that immunoglobulin-G (IgG) prepared from meat animal species, most frequently used in sausages could be used to raise antiserum and which could be made mono specific by absorbing with cross reacting antigens. Serum could detect the species specific meat (pig) from a mixture of three meat types (in sausages) by immunodiffusion methods.

Fugate and Penn (1971) recommended agar gel immunodiffusion technique for identification of animal species and observed that eleven out of twelve mixed tissue samples were identified by appearance of precipitin lines.

Ramadass (1972) found gel diffusion test to be quite useful in identification of meats of closely related species.

Kraack (1973) used a Micro-Ouchterlony method for determining soya protein, caseinate and egg white added to meat products.

Hauser et al. (1974) used immunodiffusion method to detect fresh and frozen egg, egg white, whole egg powder, soya protein and caseinate in meat product.

Munday et al. (1974) applied a immunodiffusion test for the differentiation of meats of kangaroo, bennetts, wallaby, deer, sheep and cattle.

Heever and Marais (1975) observed species specific difference of raw, heated (70 to 95 ^{o}C) and air dried malted ostrich meat from chicken, turkey, horse meat and beef by applying immunodiffusion test in agar gel.

Pandey and Pathak (1975) stated that the sera produced by the cross-immunization against alum-precipitated serum was species specific forming three lines with homologous species meat extract and did not react with heterologous species meat extracted antigen. They also reported that the absorption of rabbit antiserum with hetero species tissue had not only removed the non specific antibodies but also lowered the species specific reactivity of the serum due to absorption of antibody molecule.

Hayden (1979) raised antisera in rabbits to myoglobins from ovine, porcine and equine muscles and used sera to determine the presence of flesh from these species in beef products by agar gel diffusion technique.

Sherikar et al. (1979) observed cross reactions in number of precipitation lines with antigens of various food animals, only the antiserum raised for poultry was specific, forming three precipitation lines.

Tagore et al. (1977) prepared hyper immune sera against buffalo and cow meat in rabbits and absorbed it with heterologous cross reacting antigens. The sera so absorbed could detect aqueous cow and buffalo meat extracts by double immunodiffusion test.

Bakshi (1981) could not be able to get any reaction between antigens and heterologous antisera. Anti-buffalo, anti-camel, anti-deer and anti-goat sera raised in sheep were specific forming one precipitin band.

Ramadass and Misra (1983) applied agar gel diffusion test for differentiation of meat of different species of animals. They reported that bullock antiserum gave two distinct precipitin lines against its homologous antigen and with goat, sheep and buffalo antigens, it showed two indistinct bands. This antiserum did not react with antigens of pig and poultry. Buffalo antiserum with its homologous antigen gave a single distinct precipitin line, while it showed cross-reaction with sheep, pig and poultry antigen.

Mageau et al. (1984) conducted agar gel immunodiffusion test by using stabilizing reagent on paper discs for identifying the bovine origin of raw body tissues. The only species which cross reacted were the American bison and water buffaloes.

Sherikar *et al*. (1988) recommended the use of specific sera (absorbed) for identification of species of origin of meats in case of closely related species such as cattle and buffalo.

Tao and Poumeyrol (1989) tested IgG's from samples of meat from six different species (beef, mutton, pork, horse, rabbit and chicken) against antisera from the same species. The method was shown to identify meat simply, rapidly and reliably even in presence of mixed samples from the different species. The appearance of the precipitation lines is enhanced by the addition of polyethylene glycol to the gel.

Jain (1993) obtained a number of precipitation bands with meat antigens and unabsorbed antisera of various avian species. Antisera of all the species cross-reacted except that of peacock which did not reacted with gola pigeon antigen. Using absorbed antisera with homo and heterologous antigens, species specific reaction was observed only with homologous meat extracts with reduced number of faintly visible precipitation bands.

Kirrane and Robertson (1968) studied the antigenecity of rat skin (collagen). They used double diffusion technique to detect precipitating antibody. Antiserum of high micro-complement (C') fixing and agglutinating activity did not contain any precipitating antibody as shown by double diffusion in agar gel. For this, they concluded that precipitating antibody may not have been formed or the optimal conditions for its demonstration may not have obtained.

Lloyd et al. (1979) detected presence of IgM in skin washings of cattle by radial immunodiffusion against rabbit anti-bovine IgM globulin.

Purohit et al. (1982) applied double gel diffusion test for immunological identification of wool keratins of sheep.

2.5. Agar Gel Electrophoresis

Bennet and Boursnell (1962) suggested use of barbitone buffer with an ionic strength between 0.025 and 0.05 moles because this strength offered the best compromise for most buffering agents of stable pH, rapid electrophoretic migration without undue heating of the electrophoretic medium and protection against exceptional distortion.

Billon et al. (1980) discussed at length about the application of electrophoresis in detection of the species of origin of meat. He presented electrophoretograms of muscle proteins of food animals including horse, donkey, wild bear, red deer, fellow deer, reindeer, rabbit, rat, cat and dog.

Abdallah et al. (1983) studied the electrophoretic patterns of the sarcoplasmic proteins of fresh and frozen (-40^{o} C) meats of buffalo, cattle, camel and sheep. They noticed variations in distribution, intensity and number of electrophoretic bands among the species studied.

Aguiari and Parisi (1985) applied electrophoresis to sarcoplasmic protein and its esterase enzymes. The technique enables goat meat to be distinguished from sheep meat and chamois meat from springbok meat.

Slattery and Sinclair (1983), King (1984) and Slattery *et al*. (1984) used isoelectric focussing technique for identification of origin of meats. Sherikar *et al*. (1986) also used this technique and observed 11 and 10 fractions, respectively in cow and buffalo.

Jain (1993) obtained a characteristic band pattern for each avian species meat antigen with marked anodic and cathodic mobility.

Lahiri *et al*. (1983) obtained agar gel electrophoretic patterns of protein fractions of skin extract of a fish (*Heteropneustes fossilis*) and observed five fractions in all.

Using a modified disc electrophoresis technique, Matoltsy and Matoltsy (1963) identified two soluble proteins in hair and five in stratum corneum scrapped from the leg in humans.

Shechter *et al*. (1969) in their electrophoretic studies on hair keratins obtained 9, 14, 13, 9, and 10 distinct protein fractions in humans, monkey, dog, guinea pig and rabbit respectively.

Simonsen (1971) obtained different electrophoretic patterns of bovine and canine hair. He stated that banding of bovine pattern (from the cathode) was weak, weak, dense, weak and dense.

Purohit *et al*. (1983) characterized the keratins of sheep wool by using polyacrylamide gel electrophoresis and obtained three to four components.

2.6. Immunoelectrophoresis

Poulik (1952) conducted a two step technique of immunoelectrophoresis where he characterized the electrophoretic pattern of the putrefied diptheria toxoid on a filter paper. He embedded the toxoid under a layer of agar gel and then over laid upon the gel layer a second piece of filter paper soaked with antiserum and observed a precipitin arc between the filter paper strips in the agar gel.

Grabar and Williams (1953) carried out the modification of agar gel electrophoresis to develop the method for immunoelectrophoresis in which the electrophoresis and subsequent double diffusion precipitation reactions were performed simultaneously in the same slab of agar gel.

Scheidegger (1955) modified the technique of immunoelectrophoresis to carry out it on microscopic slides.

Williams and Grabar (1955) and Martin *et al*. (1955) used the word "immunoelectrophoresis" for the first time in technical papers and reported its utility in analysing human serum.

Grabar (1959) recommended that after electrophoresis the agar strips should be placed in a suitable buffer in order to obtain the optimum conditions before charging the reactants.

Bubloz (1962) observed a single line resulting from traces of serum in the meat extract during the identification of beef and horse meat by using immunoelectrophoresis technique.

Durand and Schneider (1962) used immunoelectrophoresis technique for differentiation of meat by using meat juices of cattle, horse, donkey, sheep, goat and camel and species-specific immune serum. Antigen and antibody precipitin arcs were observed in reacting system which were similar but fewer in number and of less intensity than those of blood serum.

Ornsttein (1964) reported best resolution in the immunodiffusion step of immunoelectrophoresis by allowing to react the antiserum with antigens as soon as possible i.e. by electrophoresing at maximum speed and applying antiserum soon after the electrophoretic run.

Sinell and Mentz (1969) recommended the immunoelectrophoresis as most useful test for serological identification of animal meat species.

Bakshi (1981) obtained one anodic and four cathodic precipitation bands in both camel and buffalo meat in immunoelectrophoresis.

Ramadass and Misra (1981) used immunoelectrophoresis for the differentiation of meats of bullock, buffalo, goat, sheep, pig and chicken. Muscle antigens of pig and chicken reacted characteristically with its homologous system. Members of bovidae family (bullock, buffalo, goat and sheep) cross reacted, but the nature and position of precipitin arcs were specific for each species.

Raut *et al*. (1988) used counter immunoelectrophoresis technique of Hari Babu *et al*. (1987) to determine species of origin of meat and meat food product heated below 80 ^{o}C.

Simonsen (1976) obtained S-carboxymethyl keratin (SCMK) from hair of man, cattle, horse, dog, cat, pig, sheep and goat. Their antigenic heterogenecity was investigated by immunoelectrophoresis. Identification of single hair and differentiation from closely related ruminant species was accomplished by indirect haemagglutination inhibition test.

Jain (1993) separated antigenic fractions of meats of avian species in agar gel under electric field and then precipitated them with homologous antiserum and observed definite number of precipitin arcs for each species.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study was conducted keeping in view of identification of antigenic determinants of meat, skin and hair of the animal species. Further, the cow whose slaughter is banned in India from religious point of view was also included in this study. Serological techniques were adopted for speedy identification of meat, skin and hair in the laboratories.

3.1. Animal Species Investigated

The following animal species were taken under the proposed study for preparation of meat, skin and hair antigens and raising their antisera.

1. Cow - *Bos indicus*
2. Buffalo - *Bubalus bubalis*
3. Camel - *Camelus dromedarius*

3.2. Collection of Samples

3.2.1. Meat

A pool of skeletal muscles of thighs, arms, neck from five apparently healthy carcasses of cow, buffalo and camel dying of surgical ailments in our College Clinics were collected and stored under frozen state in deep freeze at -20^{o} C.

3.2.2. Skin

Skin flaps from five apparently healthy carcasses of cow, buffalo and camel were taken from various body parts after complete removal of hairs by shaving.

3.2.3. Hair

Lumps of hair from various body parts of five apparently healthy cow, buffalo and camel were clipped and collected in clean polythene bags. Hairs were washed with soap and water and stored after drying.

3.3. Preparation of Antigens

3.3.1. Meat and skin

The tecnhique of Ginsberg (1948) as modified by Jain (1993) was followed for preparing phosphate buffer saline (pH 7.5) extracts of meat and skin of the animal species under study.

Fresh and frozen skeletal muscles and skin of five animals of respective species were pooled and sliced with the help of sterilized scissors and forceps in small bits separately and minced. The minced meat and skin were homogenized in warring blender in equal weight/volume (w/v) of phosphate buffer saline solution (pH 7.5, 0.01M, Appendix-I). The homogenized meat and skin were then transferred into the respective sterilized plastic tubes. The tubes were then dipped into liquid nitrogen jar for one minute and thawed in water at 37 ^{o}C. This procedure was

repeated for five times for each sample of meat and skin. The samples so treated were centrifuged at low speed for 30 minutes at room temperature. The supernatant was transferred into another centrifugal tube and centrifuged again at 10,000 rpm for 30 minutes. The clear supernatant material was taken into screw capped tubes and two drops of thiomersal (0.02%) was added. The tubes were then stored in deep freeze at -20^{o} C till further use.

3.3.2. Hair

The technique of Purohit *et al*. (1983) with slight modifications was followed for preparing lysate of hair samples of the animal species under study.

About 500 mg of washed hair samples of cow, buffalo and camel each were taken. Their grease was removed by washing the samples with warm carbon tetrachloride in separate sterilized petridishes. The hair samples were hydrolysed by adding 20ml of 10 per cent NaOH. After 24 hours the lysate of the three hair samples was dialysed against phosphate buffer saline solution pH 7.5 for three days. The dialysed hair lysate was then transferred into the respective plastic centrifuging tubes. The tubes were then dipped into liquid nitrogen jar for one minute and thawed in water at 37^{o} C. This procedure was repeated for five times for each samples. The samples so treated were centrifuged at 5000 rpm for 1 hour. The supernatant was concentrated to 3ml by freeze drying and was taken in screw capped storage tubes and two drops of thiomersal (0.02%) was added to them. The tubes were kept in deep freeze at -20^{o} C till further use.

3.4. Estimation of Protein Contents

3.4.1. Principle

The protein content of immunizing antigens were determined through Lowry's (1951) method of protein estimation. This method is based on the linear relationship between the amounts of tyrosine in protein and colour intensity. The protein content was estimated from optical density as measured by a spectrophotometer.

3.4.2. Reagents

Reagents used for this method of protein estimation are given below and their composition is given in Appendix-I.

1. Sodium hydroxide solution (5N).
2. Saturated tyrosine solution (0.02%).
3. Folin-Ciocalteu reagent (1N).
4. Tyrosine standard.
5. Blank solution.

3.4.3. Procedure

The 0.2ml of test sample of meat antigen was pipetted into a 50ml volumetric flask. To this, 25 ml of double glass distilled water, 2ml of 5N sodium hydroxide and 3ml of Folin-Ciocalteu reagent were added and the total volume was made to 50ml by adding glass distilled water.

The test solution was allowed to stand for five to ten minutes and optical density was recorded with UV-VIS Spectrophotometer-108 (Systronics) against blank solution at 660 millimicron absorbance. Total gram per cent protein content of the samples was calculated as follows :

$$\text{Protein Content} = \frac{\text{ODu}}{\text{ODs}} \times 6.4 \text{ gram per cent}$$

Where,

ODu is optical density of test sample,

ODs is optical density of standard tyrosine and

6.4 is the constant factor recommended by Greenberg (1929).

3.5. Production of Anti-Meat, Skin and Hair Sera

3.5.1. Experimental animals

Eighteen apparently healthy New Zealand White albino rabbits, two for each test sample weighing individually about 1500 gram to 2000 gram were used for production of rabbit anti-species sera against meat, skin and hair antigens of cow, buffalo and camel. All the rabbits used in this study were maintained under similar practices of feeding and management throughout the experimentation.

3.5.2. Immunization

Nine groups with two albino rabbits in each were constituted for raising antiserum against meat, skin and hair antigens of cow, buffalo and camel.

Antiserum was produced by giving four injections of antigens. Each antigen was emulsified with equal amount of Freund's incomplete adjuvant (Difco Labs) and one drop of Gentamicin (4000 I.U.) was also added to it. In rabbits an injection containing 40mg of protein of each emulsified antigen was given subcutaneously in four split doses of 10mg at flank region. Simultaneously, 0.2ml of injection "Avil" (Pheniramine maleate) was also given intramuscularly to prevent occurrence of anaphylactic shock. Each rabbit received a series of inoculation given in Table 1 to yield anti-species serum against test antigens of meat, skin and hair from respective species.

Table 1 : Immunization schedule for production of anti-sera.

Inoculation	Periodicity in days	Dose/rabbit mg antigen	Route of injection
First	0	10	s/c
Second	3rd	10	s/c
Third	6th	10	s/c
Fourth	16th	10	s/c

Note : Each rabbit was given 40mg of protein receiving 10mg at each inoculation.

3.5.3. Collection of Antisera

3.5.3.1. Bleeding

The blood from rabbits was collected after four days of the last injection i.e. on the 20th day of immunization by cardiac puncture in sterilized tubes.

3.5.3.2. Separation of serum

After collection of blood, the test tubes were kept in slanting position and blood was allowed to clot. After attaining a firm clot, the test tubes were kept in the incubator at 37°C for one hour. The clot was separated from the walls of the test tubes with the help of a sterile stainless steel wire. These tubes were then kept in the refrigerator for overnight for complete oozing of the serum from the clotted blood. On next day, the serum was separated in the sterilized centrifugal tubes and were centrifuged at 3000 rpm for 30 minutes to eliminate blood cellular components including platelets. The supernatant clear straw coloured serum was collected in 10ml amounts in sterile tubes and two drops of thiomersal (0.02%) was added. The tubes were later stored in a deep freeze at -20°C till further use.

3.6. Absorption of Antisera with Cross-Reacting Antigens

For this purpose procedure as described by Warnecke and Saffle (1968) was followed. Immunoabsorption was carried out since the antisera cross-reacted other species. For this small quantities of freeze dried cross-reacting antigens were added to the antiserum and mixed thoroughly by avoiding froathing. The tubes were incubated for five hours at 25°C and then at 4°C for 16 hours with intermittent agitation. These tube were then centrifuged at 5000 rpm for 20 minutes to sediment the precipitated cross-reacting antibody-antigens complexes and the supernatants were collected. Concentration of the absorbed

antisera was carried out by using lyphollizer and the sera thus concentrated were stored at -20 °C after adding thiomersal (0.02%) as preservative.

3.7. Serological Tests

3.7.1. Preparation of agar gel slides

Fresh microscopic slides were soaked in absolute alcohol and on the day of the test, the slide were dried and cleaned with muslin cloth. The clean glass slides were placed on a table with a leveled base to facilitate the uniform spread of 1.5 per cent agar gel medium (Appendix-I).

With the help of a pipette, 3.5ml molten agar gel cooled to 50 °C was poured over the glass slides (avoiding any air bubble). Prior to this, five drops of thiomersal (0.02%) were added to the molten agar. After solidification of gel, the slide were placed over a pre-drawn pattern on a graph paper. Wells, trough and slot of desired size were punched as per the requirement of the test to be applied i.e. double gel immunodiffusion test, agar gel electrophoresis and immunoelectrophoresis.

3.7.2. Double gel immunodiffusion test

The technique advocated by Mansi (1958) was followed.

3.7.2.1. Homologous-reacting system

On the prepared agar gel slides, wells were punched out at a distance of 6mm with the help of a metal template having an internal diameter of 5mm. A total of six wells one each for meat, skin and hair antigens of one species and one each for rabbit antiserum against the respective antigens were punched. The agar from punched wells was removed by suction under negative pressure with the help of pasteur pipette. The bottom of the wells were then sealed with a drop of molten agar.

Test Procedure :

Using the technique of Crowle (1973) the meat, skin and hair antigens of cow were filled in left side wells (A) of a slide and antiserum of the respective antigen in the right side well of the same slide (As). The test was performed for buffalo and camel meat, skin and hair antigens and antisera in the similar manner. The antigens and antisera were allowed to react by incubating the slides by keeping in a refrigerator at 4-8 ^{o}C in a moist chamber for 24 hours. Refilling of the antigens and antisera in the respective wells was repeated after 24 hours. On fifth day, the reaction was observed under oblique transmittent light for studying the pattern of precipitation bands developed in between the wells charged with antigens and antiserum.

3.7.2.2. Homologous and heterologous reacting system (cross-reactivity)

The test was carried out using both unabsorbed and absorbed antiserum with one homologous and eight heterologous antigens.

On the prepared agar gel slides nine wells (3mm diameter each) were punched out at a distance of 5mm from the centre of the central well (5mm diameter) in a circle with the help of a metal template of desired diameter. The agar from punched wells was removed by suction under negative pressure with the help of pasteur pipette. The bottom of the wells were then sealed with a drop of molten agar.

Test Procedure :

The central well was filled with the antiserum and the nine peripheral wells were filled with meat, skin and hair antigen of camel, buffalo and cow respectively in clockwise direction using technique of Crowle (1973). The test was performed for all the nine antisera (unabsorbed and absorbed) in similar manner in their respective slides. The antigens and antisera were allowed to react by incubating the slides by keeping in refregerator at 4-8 ^{o}C in a moist chamber for 24 hours. After that refilling of the antigens and antisera in the respective wells was done. On fifth day, the reaction was observed under oblique transmittent light for studying the pattern of precipitation bands developed in between the wells charged with antigens and antiserum.

3.7.3. Agar gel electrophoresis

Five samples, each of meat, skin and hair antigens of cow, buffalo and camel were processed separately in this test.

3.7.3.1. Technique

For the characterization of meat, skin and hair antigens of cow, buffalo and camel, agar gel electrophoresis was carried out by following the technique of Wieme (1959).

3.7.3.2. Reagents

Reagents used for agar gel electrophoresis are given below and their composition is given in Appendix-I.

1. Veronal buffer (0.05M), pH 8.6
2. Agar gel medium (1.5%)

3.7.3.3. Procedure

On the prepared agar gel slides, with the help of a Whatman's filter paper no.1 strip (4mm wide), a slot was made at the centre of each slide.

Setting of the electrophoretic apparatus :

The electrophoretic apparatus made from acrylic polymer sheet was used. Veronal buffer of pH 8.6 and 0.025M molarity was filled in both the chambers of this instrument. The molten agar filled in agar chambers was allowed to solidify. The continuity of agar chamber with buffer was maintained through the drilled holes in partition wall between the agar chamber and buffer tank.

Procedure of electrophoretic run :

After setting the electrophoretic apparatus, the meat, skin and hair antigens of cow, buffalo and camel were charged in the slot in the respective slides. By using a needle tip a mark of tracking dye (Bromophenol blue) was made at one corner near the slot. The slides were then placed in inverted position on agar blocks in electrophoretic apparatus for the continuity of the electric current. The electrodes were connected to a power pack and a direct current (DC) of 50mA with potential difference of 120 volts was allowed to run till the dye reached the anodic end of the agar gel slides (in approximately 35-50 minutes). During electrophoretic run the apparatus was kept cool by keeping it in the refrigerator.

Fixation and staining of the slides :

After electrophoretic run, the slides were removed and fixed in two per cent glacial acetic acid. The slides were then air dried by placing filter papers soaked in two per cent glacial acetic acid solution and then stained with protein stain (0.1% amido black).

Observation :

Using Systronics Densitometer-201 and green filter, the optical densities of protein fractions of stained slides were measured. Graphs were plotted for each antigen on

the basis of optical densities so measured and percentage of the various fractions were recorded. The numbers were assigned to each fraction by following the procedure of Vesselinovitch (1959).

3.7.4. Immunoelectrophoresis

3.7.4.1. Technique

The technique of Wieme (1959) was followed for characterization of anodic and cathodic antigens of meat, skin and hair of cow, buffalo and camel respectively, by immunoelectrophoresis.

3.7.4.2. Reagents

Reagents used for immunoelectrophoresis are given below and their composition is given in Appendix-I.

1. Veronal buffer (0.05M), pH 8.6
2. Agar gel medium (1.5%)

3.7.4.3. Procedure

On a prepared agar gel slides a well was punched out at the centre of the slide with the help of a metal template having an internal diameter of 3mm. A long trough of 6cm x 2 mm was made at side of the well at a distance of 5mm with the help of a cutter fitted with razor blade. The agar was remove from the punched well and the long trough by back suction and the bottom of the well and long trough were sealed with molten agar.

Setting of the electrophoretic apparatus :

The electrophoretic apparatus made from acrylic polymer sheet was used. Veronal buffer of pH 8.6 and 0.025M molarity was filled in both the chambers of this instrument. The molten agar filled in agar chambers was allowed to solidify. The continuity of agar chamber with buffer was maintained through the drilled holes in partition wall between the agar chamber and buffer tank.

Procedure of electrophoretic run :

After setting the electrophoretic apparatus, the meat, skin and hair antigens of cow, buffalo and camel were charged in the well in the respective slides. By using a needle tip a mark of tracking dye (Bromophenol blue) was made near the wells. The slides were then placed in inverted position on agar blocks in electrophoretic apparatus for the continuity of the electric current. The electrodes were connected to a power pack and a direct current (DC) of 50mA with potential difference of 120 volts was allowed to run till the dye reached at the anodic end of the agar gel slides (in approximately 35-50 minutes). During electrophoretic run the apparatus was kept cool by keeping it in the refrigerator.

Step of immunodiffusion :

After electrophoretic run the slides were removed and the corresponding antisera were charged in the long trough. The slides were then placed in the humid atmosphere in moist

chamber at 8°C, after 24 hours the troughs were recharged with corresponding antisera and again placed in the humid atmosphere. On fifth day the slides were examined under oblique transmittent light to observe the development of precipitation arcs and results were noted.

3.8. Drying and Staining of the Gel Slides

3.8.1. Drying of slides

To prepare permanent stained slides of double gel immunodiffusion and immunoelectrophoresis, the slides were kept immersed in 2 per cent physiological saline solution (NSS) for a period of 24-36 hours. The frequent changes of NSS was done in order to remove the soluble non-reacting constituents i.e. antigen or antibody etc. The slides were finally washed by dipping them in distilled water to remove excess amount of salt. After washing, the slides were covered with wet filter paper and kept for drying at 37°C. Precautions were taken, not to leave any air space while placing the wet filter paper on gel surface.

3.8.2. Staining of slides

The dried and fixed slides of immunodiffusion, agar gel electrophoresis and immunoelectrophoresis were stained as per the technique of Richard et al. (1958) by dipping them in 0.1 per cent amido black, a protein stain solution (Appendix-I) for 15 minutes and destained with several changes of destaining solution (Appendix-I) till the excess of stain was removed and a clear background was observed. The stained slides were finally air dried at room temperature and were preserved.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

The techniques adopted for characterization of meat, skin and hair antigens of the animal species in the present study were primarily based on the basic principles of antigens and antibody reactions. The protein antigens maintained their antigenic property in stimulating the reaction in-vivo and in vitro when administered either parenterally in heterologous hosts or reacting in a visible form by precipitation test and for this the antigen and antibody were allowed to react in agar gel medium. Earlier work of various research workers cited in the texts are duly acknowledged at appropriate places, although the literature is not ardently available with reference to serological work on skin and hair of animal species.

4.1. Immunizing Antigen

In the present study the meat, skin and hair antigens were prepared by freezing and thawing and were used as immunizing antigens.

Meat extract antigens were also prepared earlier by Oswald (1953), Herran and Meliton (1961), Bubloz (1962), Warnecke and Saffle (1968), Munday et al. (1974), Sherikar et al. (1979), Bakshi (1981) and Jain (1993). Our observations are also in agreement with these workers. Further, our work is also in agreement with the earlier finding of Purohit et al. (1987) who

used freezing and thawing technique for preparation of cell membrane and flagellar antigens of Trypanosoma evansi and Jain (1993) who also used this technique for preparation of muscle antigens of nine avian species.

Our observations showed that similar technique of freezing and thawing when used, it gives satisfactory results in preparing skin and hair antigens of cow, buffalo and camel. Present work is also in collaboration with the work of Lahiri et al. (1983), who successfully used the technique of freezing and thawing for preparation of skin extract of a fish (Heteropneustes fossilis). Hair samples were dissolved in sodium hydroxide for preparing its lysate. Purohit et al. (1982) also dissolved the wool samples in the similar manner. Presently, the dissolved hair samples after processing were further subjected to freezing and thawing for preparation of hair antigen. The technique used for the preparation of hair antigens was quite satisfactory and the results are also in agreement with the work of Purohit et al. (1982).

4.2. Immunization Schedule

The immunization schedule of 0, 3rd, 6th and 16th day for immunization of rabbits with antigens in split doses of 10mg (total 40mg) protein was observed satisfactory to stimulate the precipitin antibodies. Split dosing was also used earlier by Shunmugam and Ranganathan (1972), Bakshi (1981), Ramadass and Misra (1981) (for mammalian meat antigens), Jain (1993) (for meat antigens of avian species), and Purohit et al. (1982) (for wool

antigens). From the present studies it can be concluded that such schedule can be used successfully for preparation of meat as well as skin and hair antigens.

4.3. Production of Antisera Against Meat, Skin and Hair Antigens.

In the present study albino rabbits were used to raise the antibodies against meat, skin and hair antigens of cow, buffalo and camel, and it was found to be a suitable laboratory animal for raising antisera. This is evident from Figure no. 1 to 9 and 13 to 21 which shows that antiserum so produced yielded satisfactory sharp precipitation bands in gel diffusion and immunoelectrophoresis. Evans (1957) also suggested that rabbit is the best laboratory animal for superior antibody production. Purohit *et al*. (1982) also raised antisera against sheep wool lysate successfully in rabbits and further, Jain (1993) also found that rabbit is most suitable for raising anti-meat sera against nine avian species.

4.4. Protein Contents of Immunizing Antigen

The total protein content of antigens was determined by Lowry's (1951) method. The results calculated by spectrophotometric observations are shown in Table 2. It is evident that the total protein contents of antigens of meat skin and hair of animal species used in this investigation ranged from 0.57gm per cent to 1.39gm per cent. The buffalo meat antigen depicted highest value of 1.39, whereas antigen of cow hair showed a least quantum of 0.57gm per cent. However, the protein content of meat antigens of avian species was found to be 5gm per

cent approximately by Jain (1993) which is quite higher in comparison to meats of animal species used in our study. From the present investigation it can be concluded that hair antigens of cow, buffalo and camel contains less amount of gram per cent protein in comparison to meat and skin antigens.

4.5. Double Gel Immunodiffusion Test

4.5.1. Homologous reaction with unabsorbed antisera

When meat, skin and hair antigens of cow, buffalo and camel were allowed to react with their homologous unabsorbed antisera in double gel immunodiffusion test, they showed 4, 3, 1; 4, 3, 1; 7, 2 and 2 precipitation lines, respectively.

The results of the test are summarized in Table no. 3 and represented in Fig. 1,2 and 3. The precipitation reaction with a maximum of 7 precipitation bands was observed in camel meat, followed by 4 each in cow and buffalo meat, 3 in cow and buffalo skin, 2 in camel skin, 1 each in cow and buffalo hair and 2 in camel hair. The object of gel diffusion is to bring together, through diffusion, optimal concentrations of antigen and antibody to form visible bands of precipitation. The pattern of precipitin line indicates the characteristics of the antigenic component, as to their diffusion rates and molecular weights. The antigenic component having a high rate of diffusion formed precipitin line near the antiserum well, and so bends towards this well. The diffusion component with lower diffusion rate formed precipitin lines near the antigenic well. The components which had the same rate of diffusion as that of antiserum formed a straight line without exibiting any bending effect.

Table 2 : Total protein content of immunizing antigens

S.No.	Type of species and antigen	Total protein content (gram per cent)
1.	Cow meat	1.16
2.	Cow skin	0.89
3.	Cow hair	0.57
4.	Buffalo meat	1.39
5.	Buffalo skin	1.13
6.	Buffalo hair	0.67
7.	Camel meat	1.27
8.	Camel skin	1.06
9.	Camel Hair	0.96

For meat, the results of the study are in accordance with Heever (1962), who found that raw meats could be identified qualitatively, based on the number of precipitin lines and their characteristic pattern. In the present study, four precipitation lines were observed in the reaction of cow and buffalo meat antigens and their respective antisera which is in full agreement with the findings of Ramadass and Misra (1983) and Sherikar et al. (1988). While findings of Bakshi (1981) shows that only one precipitation band could be observed in the reaction of meat antigens of buffalo and camel, when their antisera was raised in sheep. In contrast to this study our result show better immunological response probably due to freezing and thawing method used for preparation of antigen as well as using rabbit for raising antisera.

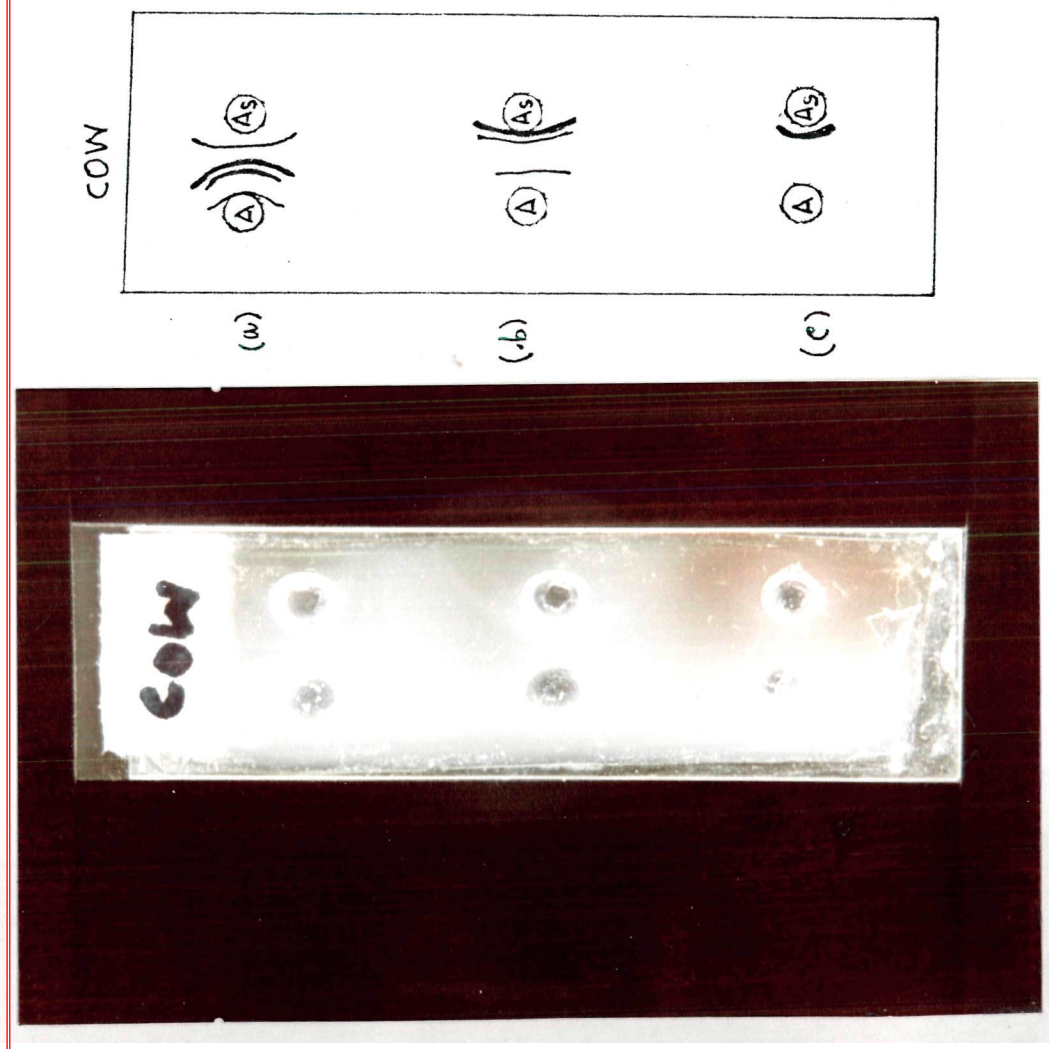
The antigen-antibody reaction of skin was quite satisfactory in case of cow, buffalo and camel as evident from the results shown in Fig. 1(b), 2(b) and 3(c). This is in agreement with the work of Lloyd et al. (1979).

Kirrane and Robertson (1968) used salt soluble antigen of rat collagen and failed to detect any precipitating antibody by using double diffusion test. While successful detection of antibodies against skin antigens of cow, buffalo and camel in present study may be attributed to the difference in the method of preparing antigen and subsequently raising antisera in rabbits.

Table 3 : Precipitation bands obtained in double immunodiffusion test between meat, skin and hair antigens of cow, buffalo and camel with their homologous unabsorbed antiserum.

S.No.	Type of species and antigen	Number of precipitation bands
1.	Cow meat	4
2.	Cow skin	3
3.	Cow hair	1
4.	Buffalo meat	4
5.	Buffalo skin	3
6.	Buffalo hair	1
7.	Camel meat	7
8.	Camel skin	2
9.	Camel Hair	2

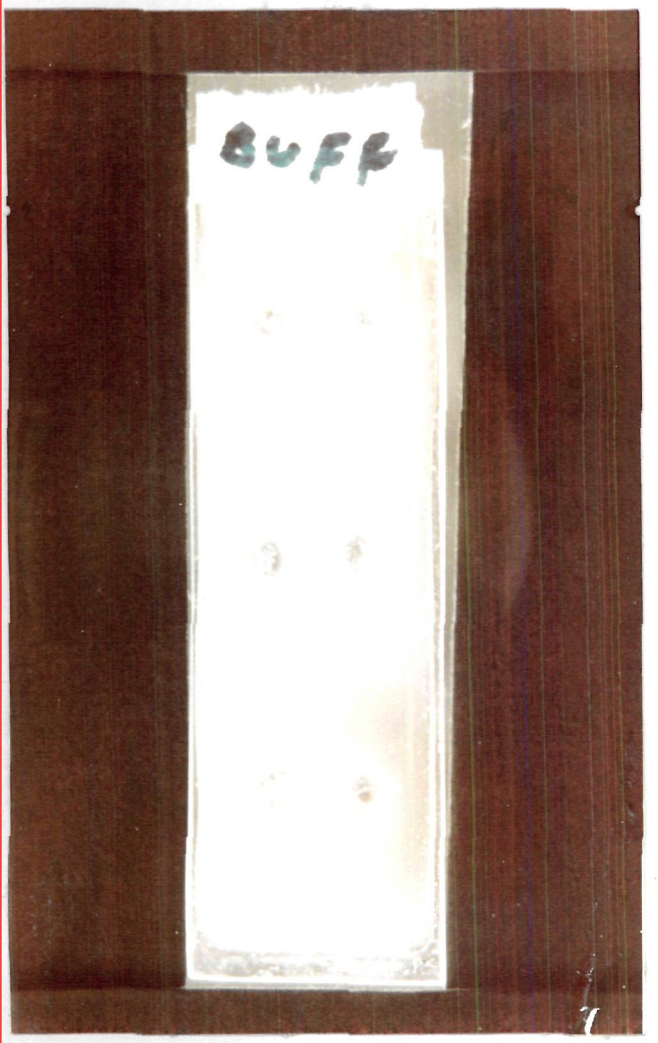
Figure 1 : Photograph (a) and schematic diagram (b) of double gel immunodiffusion test of cow's meat(a), skin(b) and hair(c) showing precipitation reactions of antigen (A) and unabsorbed antiserum (As) (unstained slides).



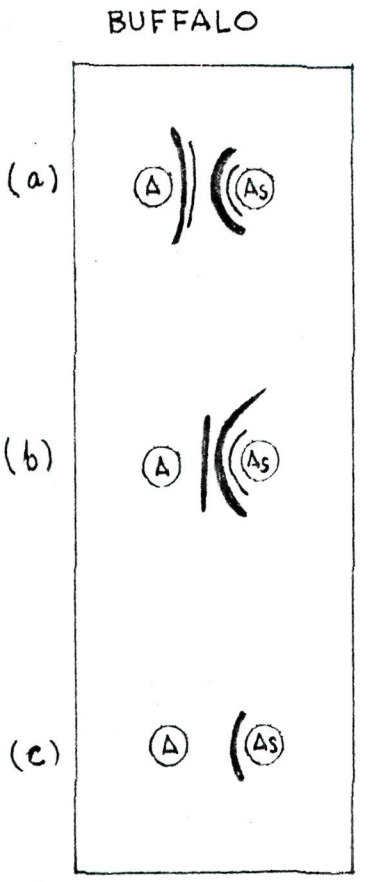
(a)

(b)

Figure 2 : Photograph (a) and schematic diagram (b) of double gel immunodiffusion test of buffalo's meat(a), skin(b) and hair(c) showing precipitation reactions of antigen (A) and unabsorbed antiserum (As) (unstained slides).

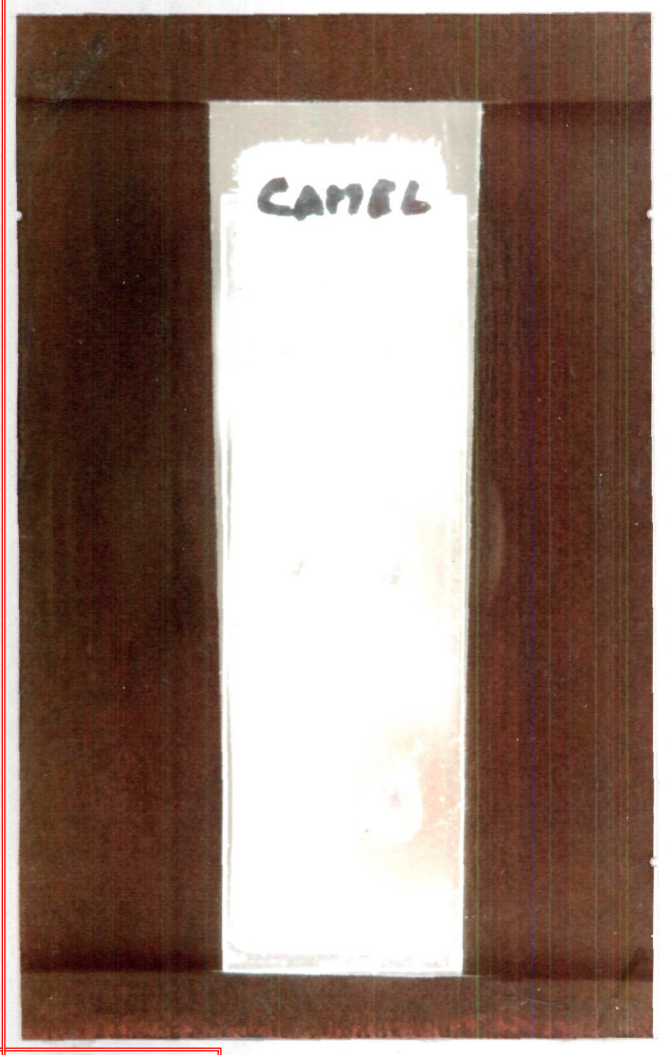


(a)

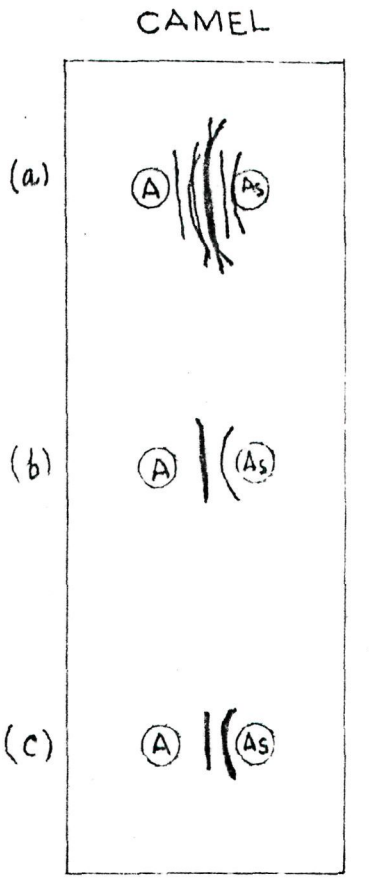


(b)

Figure 3 : Photograph (a) and schematic diagram (b) of double gel immunodiffusion test of camel's meat(a), skin(b) and hair(c) showing precipitation reactions of antigen (A) and unabsorbed antiserum (As) (unstained slides).



(a)



(b)

Results obtained in the reactions of hair antigens of cow, buffalo and camel with their respective antisera are in fully agreement with the work of Purohit et al. (1982) who identified the sheep wool keratins by using the similar technique. The main advantage of gel diffusion test lies in the detection of more than one antigen-antibody system. The test has a higher degree of sensitivity and provides better resolution in identification of components in mixtures and gives a valuable guide for purification of either antigen or antibody.

For the present work the double gel diffusion test was used as it is simple, non-expensive and provides definite results in short time which is in confirmity regarding efficacy of the test as used earlier by Sherikar et al. (1979), Bakshi (1981), Ramadass and Misra (1983) and Jain (1993).

4.5.2. Homologous and heterologous reaction (cross-reaction studies) with unabsorbed antiserum

In the present investigation the double gel immunodiffusion test was conducted to determine the antigenic correlation between meat, skin and hair of cow, buffalo and camel. By using a ten well system, the homologous and heterologous antigens were characterized by using the double gel immunodiffusion test on microscopic slides. Central well of each slide was charged with unabsorbed antiserum and the peripheral wells were charged with one homologous and eight heterologous antigens.

The results obtained are summarized in Table 4 and represented in Fig 4, 5 and 6. The precipitin lines were numbered from peripheral (antigen) wells to the central well, considering only homologous antigen and antibody reaction. The results are described as under.

4.5.2.1. Rabbit anti-cow meat serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The cow meat antiserum showed four precipitation lines when reacted with homologous meat antigen and two each with the antigen of cow skin and buffalo meat. Cross-reaction was not observed with other antigens [Fig. 4(G)].

The precipitin line number 1 and 2 were independent and were found near the antigen well. Precipitin line number 3 showed the common reaction with cow skin antigen. Precipitin line number 4 was observed near the central well.

4.5.2.2. Rabbit anti-cow skin serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The cow skin antiserum showed three precipitin lines when it reacted with homologous skin antigen, while antigens of cow meat, buffalo hair, skin and meat showed 1,1,1 and 3 precipitin lines, respectively [Fig. 4(H)].

Precipitin line number 1 and 2 were found to be independent. Precipitin line number 3 was common to antigens of cow meat, buffalo hair, skin and meat.

Table 4 : Number of precipitin lines observed in homologous and heterologous reactions in double gel immunodiffusion test using unabsorbed anti meat, skin and hair sera with meat, skin and hair antigens of cow, buffalo and camel.

Sr. No.	Type of species and antisera	Antigens COM	COS	COH	BUM	BUS	BUH	CAM	CAS	CAH
1.	Cow meat (COM)	+++ +	++	–	++	–	–	–	–	–
2.	Cow skin (COS)	+	+++	–	+++	+	+	–	–	–
3.	Cow hair (COH)	++	+	+	++	+++	+++	–	–	–
4.	Buffalo meat (BUM)	++	–	–	+++ +	++	–	–	–	–
5.	Buffalo skin (BUS)	+++	–	–	+++ ++	+++	–	–	–	–
6.	Buffalo hair (BUH)	–	–	–	–	–	+	–	–	–
7.	Camel meat (CAM)	++	–	–	+++	–	–	+++ +++ +	++	–
8.	Camel skin (CAS)	–	–	–	–	–	–	–	++	–
9.	Camel Hair (CAH)	–	–	–	–	–	–	–	–	++

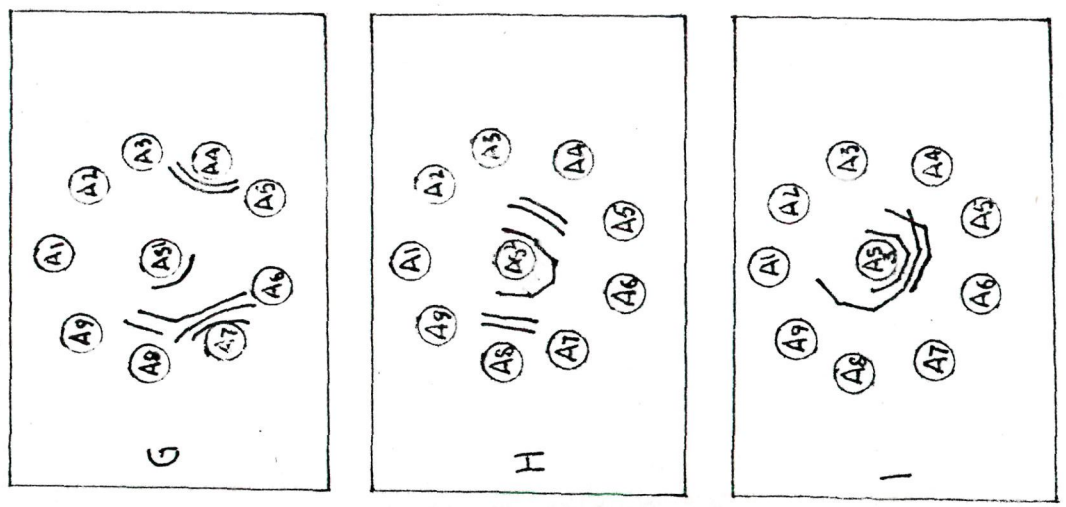
Note : + means one precipitation line
– means no precipitation line

Figure 4 : Photograph (a) and schematic diagram (b) of double gel immunodiffusion test showing cross-reactivity of antiserum of cow's meat(G), skin(H) and hair(I) with meat, skin and hair antigens of cow, buffalo and camel (unstained slides).

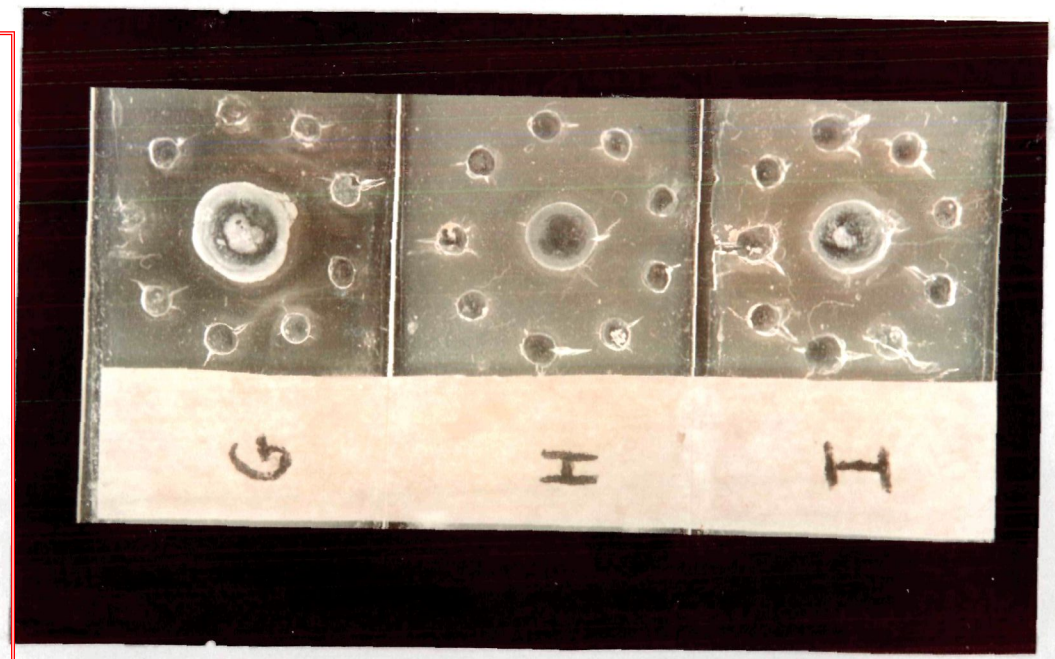
Where,

A1 - Camel meat antigen
A2 - Camel skin antigen
A3 - Camel hair antigen
A4 - Buffalo meat antigen
A5 - Buffalo skin antigen
A6 - Buffalo hair antigen
A7 - Cow meat antigen
A8 - Cow skin antigen
A9 - Cow hair antigen
As1 - Rabbit anti-cow meat serum
As2 - Rabbit anti-cow skin serum
As3 - Rabbit anti-cow hair serum

COW



(b)

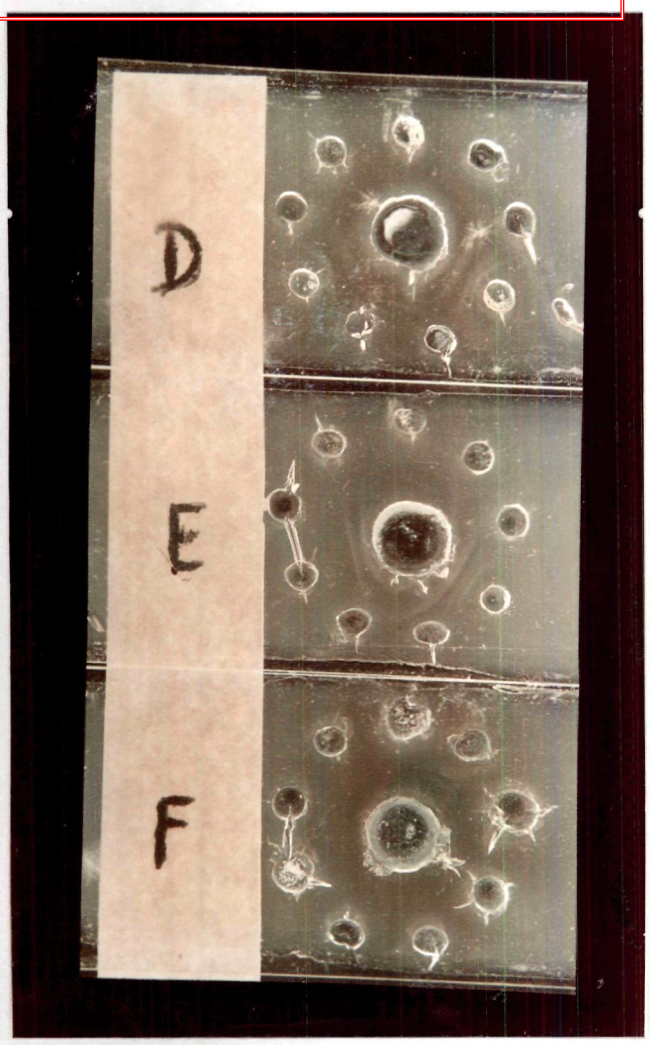


(a)

Figure 5 : Photograph (a) and schematic diagram (b) of double gel immunodiffusion test showing cross-reactivity of antiserum of buffalo's meat(D), skin(E) and hair(F) with meat, skin and hair antigens of cow, buffalo and camel (unstained slides).

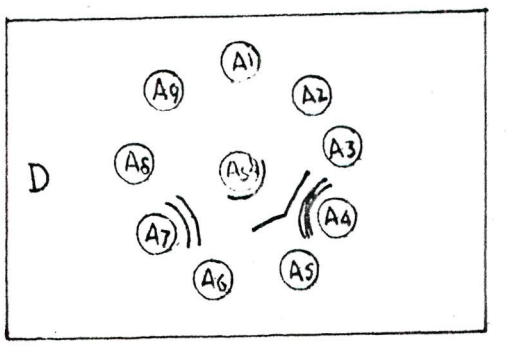
Where,

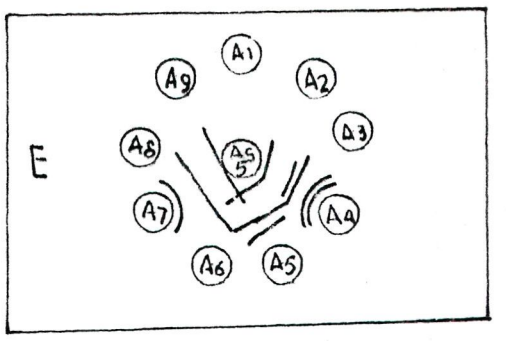
A1 - Camel meat antigen
A2 - Camel skin antigen
A3 - Camel hair antigen
A4 - Buffalo meat antigen
A5 - Buffalo skin antigen
A6 - Buffalo hair antigen
A7 - Cow meat antigen
A8 - Cow skin antigen
A9 - Cow hair antigen
As4 - Rabbit anti-buffalo meat serum
As5 - Rabbit anti-buffalo skin serum
As6 - Rabbit anti-buffalo hair serum

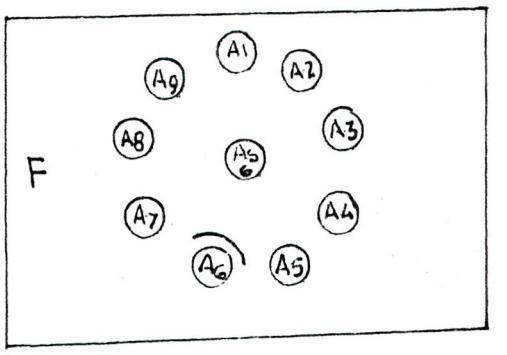

D
E
F

(a)

BUFFALO


D
A1
A2
A3
A4
A5
A6
A7
A8
A9


E
A1
A2
A3
A4
A5
A6
A7
A8
A9

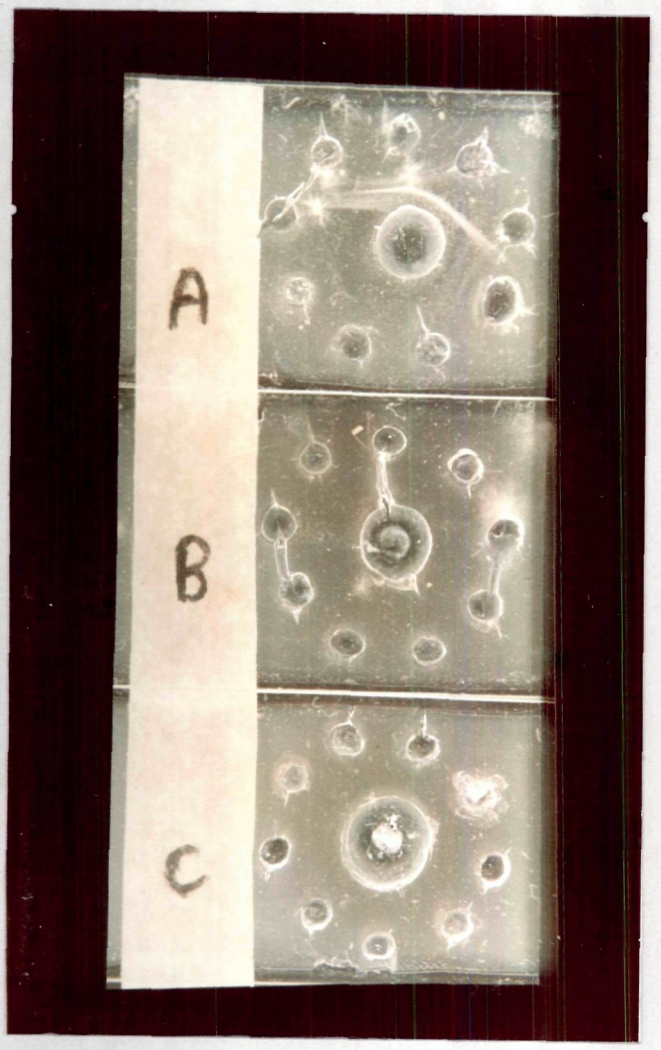

F
A1
A2
A3
A4
A5
A6
A7
A8
A9

(b)

Figure 6 : Photograph (a) and schematic diagram (b) of double gel immunodiffusion test showing cross-reactivity of antiserum of camel's meat(A), skin(B) and hair(C) with meat, skin and hair antigens of cow, buffalo and camel (unstained slides).

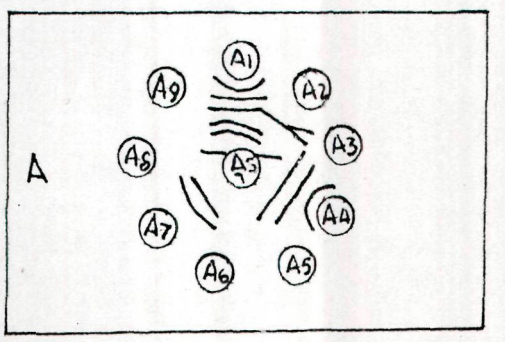
Where,

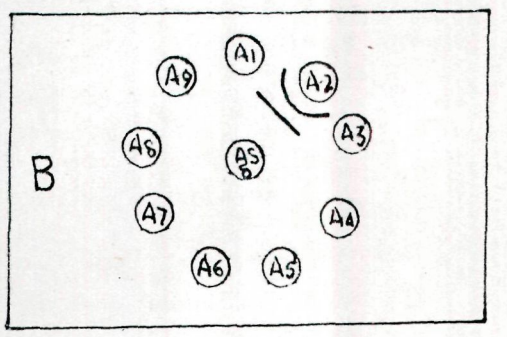
A1 - Camel meat antigen
A2 - Camel skin antigen
A3 - Camel hair antigen
A4 - Buffalo meat antigen
A5 - Buffalo skin antigen
A6 - Buffalo hair antigen
A7 - Cow meat antigen
A8 - Cow skin antigen
A9 - Cow hair antigen
As7 - Rabbit anti-camel meat serum
As8 - Rabbit anti-camel skin serum
As9 - Rabbit anti-camel hair serum

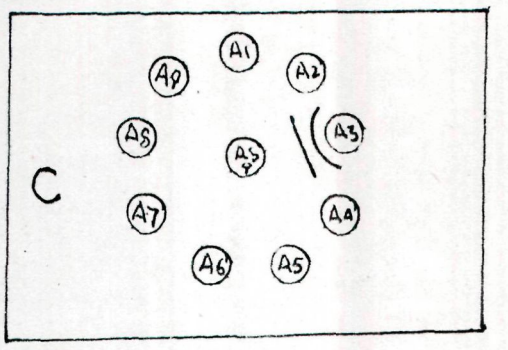

A
B
C

(a)

CAMEL


A
A1
A2
A3
A4
A5
A6
A7
A8
A9


B
A1
A2
A3
A4
A5
A6
A7
A8
A9


C
A1
A2
A3
A4
A5
A6
A7
A8
A9

(b)

4.5.2.3. Rabbit anti-cow hair serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The cow hair antiserum showed only one precipitation line when it reacted with homologous hair antigen, while the antigens of cow skin and cow meat, buffalo hair, skin and meat showed 1,2,3,3 and 2 precipitin lines, respectively [Fig. 4(I)].

Single precipitin line as observed in the reaction of cow hair antigen with its antibody, further, it was found to be common to cow skin and cow meat, buffalo hair and skin antigens.

4.5.2.4. Rabbit anti-buffalo meat serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The buffalo meat antiserum showed four precipitation lines when it reacted with homologous meat antigen, while the antigens of buffalo skin and cow meat showed two precipitin lines each [Fig. 5(D)].

Precipitin line number 1 and 2 were independent and were found near the antigen well. Precipitin line number 3 and 4 showed a common reaction with the precipitin line number 1 and 2 of buffalo skin antigen, respectively.

4.5.2.5. Rabbit anti-buffalo skin serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The buffalo skin antiserum showed three precipitation lines when it reacted with homologous skin antigen, while antigen of buffalo and cow meat showed five and three precipitation lines, respectively. Cross-reaction was not observed with other antigens [Fig. 5(E)].

In the above reaction precipitin line number 1 was independent and precipitin line number 2 was common with precipitin line number 3 of buffalo meat antigen. Precipitin line number 3 was common with precipitin line number 5 of buffalo meat antigen and it was observed very close to the central well.

4.5.2.6. Rabbit anti-buffalo hair serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The buffalo hair antiserum showed only one precipitation line which was present near the antigen well when it reacted with homologous hair antigen. Cross-reaction with other antigens was not observed [Fig. 5(F)].

4.5.2.7. Rabbit anti-camel meat serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The camel meat antiserum showed seven precipitation lines when it reacted with homologous meat antigen, while antigens of camel skin, buffalo and cow meat showed 2,3 and 2 precipitation lines, respectively [Fig. 6(A)].

Precipitin line number 1 and 2 were independent and were near the antigen well. Precipitin line number 3 and 4 were merged and were common to the two precipitation lines of camel skin antigen where they emerged in the form of a spur. Precipitation line number 5 and 6 were present near the central well and were independent. Precipitation line number 7 was present just at the periphery of the antiserum well.

4.5.2.8. Rabbit anti-camel skin serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The camel skin antiserum showed two precipitation lines when it reacted with homologous skin antigen. No cross reaction with other antigens was observed in the reaction of this test [Fig. 6(B)].

The precipitation line number 1 was present near the antigen well and was independent and precipitation line number 2 was also independent and was present between antigen and antiserum well.

4.5.2.9. Rabbit anti-camel hair serum with nine antigens of meat, skin and hair of cow, buffalo and camel.

The camel hair antiserum showed two precipitation lines with homologous hair antigen. No cross-reaction with other antigens was observed in this test [Fig. 6(C)].

Precipitin line number 1 was present near the antigen well while, the precipitin line number 2 was present between antigen and antiserum well. Both the precipitation lines were found to be independent.

In Table 4 the reaction between homologous and heterologous antisera with homologous and heterologous antigens has been summarized, which reveals that cow and buffalo meat showed cross-reaction while camel meat antisera cross-reacted with both cow and buffalo meat. Such results regarding cross-reactions between buffalo and cow meat has also been reported earlier by Pinto (1961), Singh and Yadav (1962), Pandey and Pathak (1975), Mageau *et al*. (1984) and Sherikar *et al*. (1988). Significant cross-reactivity between sheep and goat meat antigens have also been reported by Pinto (1961) and Sherikar *et al*. (1979). Our result does not collaborate with the earlier findings of Bakshi (1981) where camel meat does not give any cross-reaction with buffalo meat. The probable reason could be that in his work, antisera against camel meat was raised in sheep, being a ruminant, an immunizing antigen came from the ruminant class of animal, thus most of the non self common antigens of ruminants were eliminated.

The result of present work suggests that camel meat antisera behaved in a different pattern in comparison to reaction between cow and buffalo meat antigen. Fascinating fact observed in the present study as revealed by the results that antiserum of cow, buffalo and camel meat cross-reacted with their own skin antigens but such reaction was not observed with antigens of hairs of any of the species under study.

In the present study, skin antisera of cow showed precipitation reaction with its own antigen and antigen of meat. Further, it showed a common reaction with meat, skin and hair antigens of buffalo. The buffalo skin antisera does not behaved in the similar manner as that of cow skin antisera, as it reacted with its antigen and also with meat antigens of buffalo and cow. It is worthy to note that camel skin did not show any cross-reactivity with other eight antigens and such results can help very well in vetero-legal cases to identify the submitted skin pieces for examination.

The results of cow hair antiserum reaction showed that apart from its antigen all the other five antigens of cow and buffalo share common antigenecity, while it did not give any reaction with any of the camel antigens used in this study. It is worthy to note that buffalo and camel hair antisera reacted only with their own antigens. From the findings of present work it can concluded buffalo and camel hair can be recognised as they maintained their identity of antigenic component and thus can be used in differentiating the hair samples submitted for vetero-legal cases regarding the species under study.

Literature in reference to the cross-reactivity pattern of ruminant skin and hair antigens by double diffusion test could not be traced out and such type of studies may be helpful in solving disputed cases in the court of law.

4.5.3. Homologous and heterologous reaction with absorbed antiserum in double immunodiffusion test.

The double immunodiffusion test was conducted to determine the species-specific reactivity of absorbed antiserum with homologous antigens. For this, absorbed antiserum of each antigen was allowed to react with homologous and other eight heterologous antigens. The results obtained are summarized in Table 5 and represented in Fig. 7,8 and 9.

In the present study the reactivity of the meat, skin and hair antisera of cow, buffalo and camel was found to be reduced to some extent, but still it was mono specific and produced only homologous reaction. No cross-reactivity was observed among all the antigens except in case of camel meat antisera where, even after its absorption with all the heterologous antigens including camel skin, it showed reaction with camel skin antigen. The antiserum of buffalo and cow meat did not give any cross-reaction with their skin antigens.

The results of this study revealed that species-specific antisera could be procured for cow and buffalo meat, and skin and hair antigens of all the three species under study. The same is not concluded for camel meat antigens as its antisera cross-reacted with its skin antigen even after absorption.

Table 5 : Number of precipitin lines observed in homologous and heterologous reactions in double immunodiffusion test using absorbed anti meat, skin and hair sera with meat, skin and hair antigens of cow, buffalo and camel.

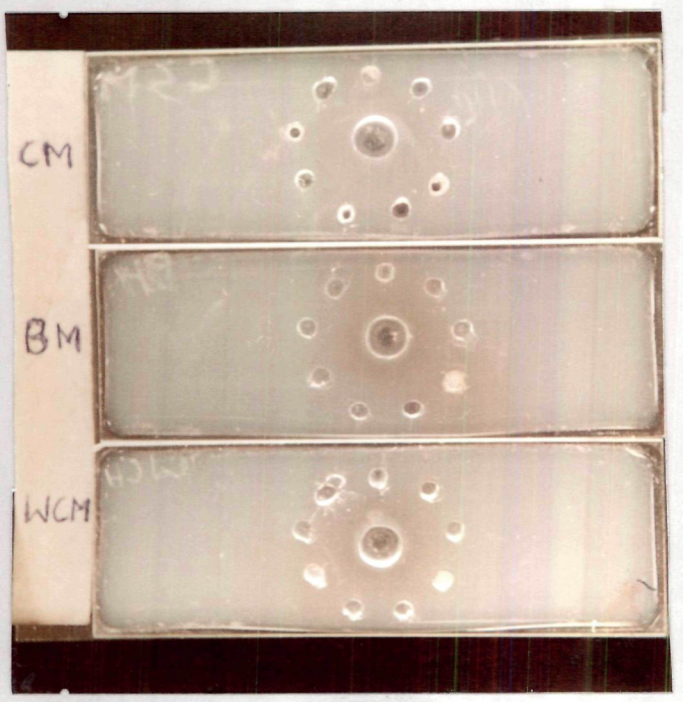
Sr. No.	Type of species and antisera	Antigens COM	COS	COH	BUM	BUS	BUH	CAM	CAS	CAH
1.	Cow meat (COM)	+	–	–	–	–	–	–	–	–
2.	Cow skin (COS)	–	+	–	–	–	–	–	–	–
3.	Cow hair (COH)	–	–	+	–	–	–	–	–	–
4.	Buffalo meat (BUM)	–	–	–	+	–	–	–	–	–
5.	Buffalo skin (BUS)	–	–	–	–	++	–	–	–	–
6.	Buffalo hair (BUH)	–	–	–	–	–	+	–	–	–
7.	Camel meat (CAM)	–	–	–	–	–	–	++	+	–
8.	Camel skin (CAS)	–	–	–	–	–	–	–	+	–
9.	Camel Hair (CAH)	–	–	–	–	–	–	–	–	+

Note : + means one precipitation line
– means no precipitation line

Figure 7 : Photograph (a) and schematic diagram (b) showing effect of immuno absorption specificity of antiserum of camel, buffalo and cow meat, central well containing absorbed antiserum against respective antigen and nine peripheral wells, the antigens (unstained slides).

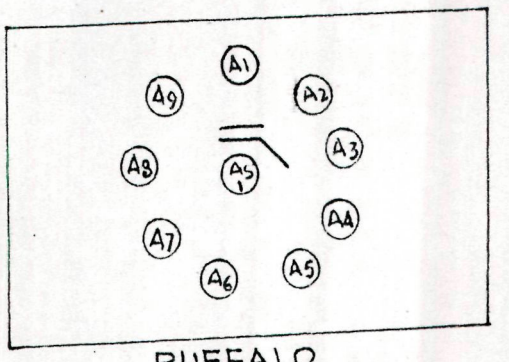
Where,

A1 - Camel meat antigen
A2 - Camel skin antigen
A3 - Camel hair antigen
A4 - Buffalo meat antigen
A5 - Buffalo skin antigen
A6 - Buffalo hair antigen
A7 - Cow meat antigen
A8 - Cow skin antigen
A9 - Cow hair antigen
As7 - Rabbit anti-camel meat serum
As4 - Rabbit anti-buffalo meat serum
As1 - Rabbit anti-cow meat serum

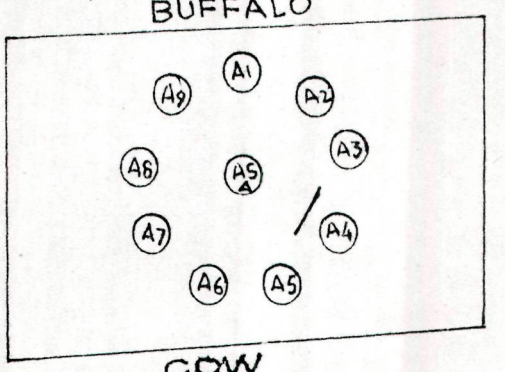


(a)

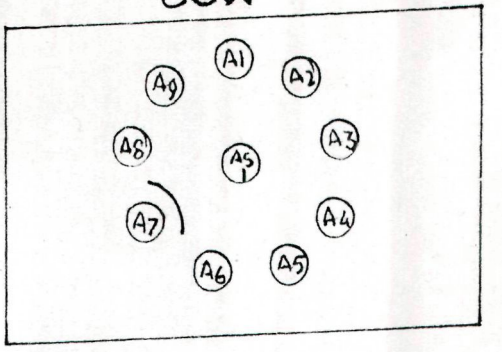
CAMEL



BUFFALO



COW



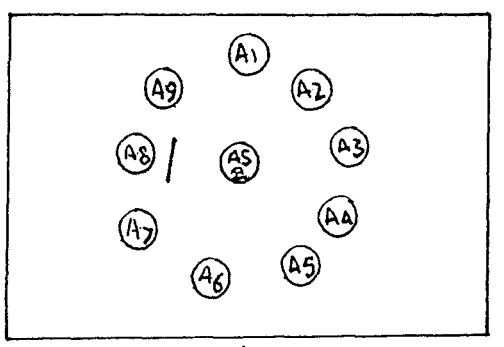
(b)

Figure 8 : Schematic diagram showing effect of immuno absorption specificity of antiserum of camel, buffalo and cow skin, central well containing absorbed antiserum against respective antigens and nine peripheral wells, the antigens.

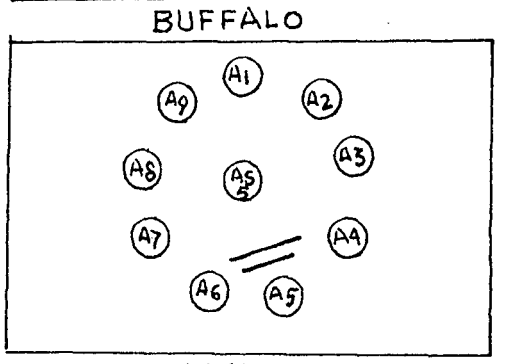
Where,

A1 - Camel meat antigen
A2 - Camel skin antigen
A3 - Camel hair antigen
A4 - Buffalo meat antigen
A5 - Buffalo skin antigen
A6 - Buffalo hair antigen
A7 - Cow meat antigen
A8 - Cow skin antigen
A9 - Cow hair antigen
As2 - Rabbit anti-cow skin serum
As5 - Rabbit anti-buffalo skin serum
As8 - Rabbit anti-camel skin serum

COW



BUFFALO



CAMEL

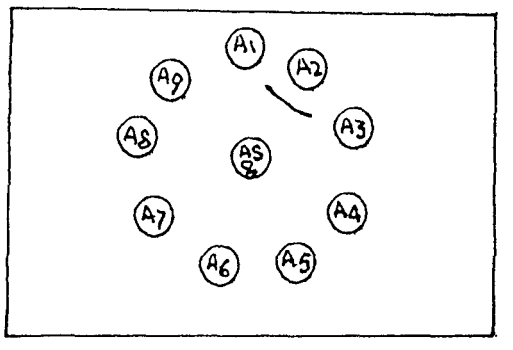
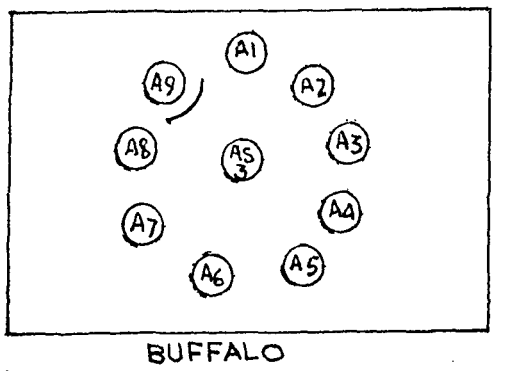


Figure 9 : Schematic diagram showing effect of immuno absorption specificity of antiserum of camel, buffalo and cow hair, central well containing absorbed antiserum against respective antigens and nine peripheral wells, the antigens.

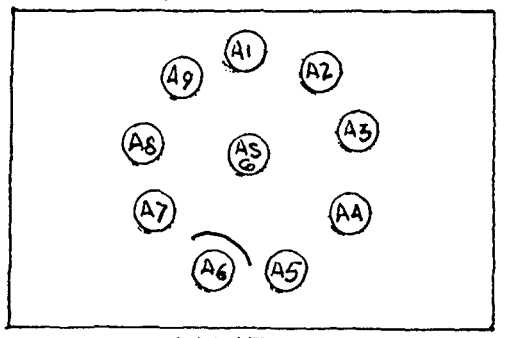
Where,

A1 - Camel meat antigen
A2 - Camel skin antigen
A3 - Camel hair antigen
A4 - Buffalo meat antigen
A5 - Buffalo skin antigen
A6 - Buffalo hair antigen
A7 - Cow meat antigen
A8 - Cow skin antigen
A9 - Cow hair antigen
As3 - Rabbit anti-cow hair serum
As6 - Rabbit anti-buffalo hair serum
As9 - Rabbit anti-camel hair serum

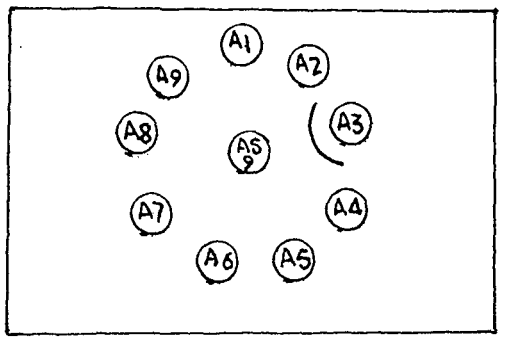
COW



BUFFALO



CAMEL



For meat antigens (reaction with heterologous meat antisera), the absorption process so applied, appeared to be in confirmation to the observation made by Pandey and Pathak (1975) and Jain (1993). The absorption of rabbit antiserum with hetero species antigens remove all the non-specific antibodies and these workers found that species-specific reactivity of antiserum was lowered due to absorption of antibody molecule and similar observations were also found in the present study.

Present findings also fall in line with the earlier findings of Tagore et al. (1977), Sherikar et al. (1988) and Jain (1993), who earlier used this technique and recommended that specific antisera is worth to use (after absorption) for identification of species of origin of meats in case of closely related species. Present findings are also in accordance with the work of Warnecke and Saffle (1968), as they also stated that after absorption of antiserum with cross-reacting freeze dried meat extract, it becomes species-specific and reacts only with homologous meat extract.

Ramadass (1972) in his study on identification of closely related species found agar gel immunodiffusion test to be quite useful. From the present study it can be concluded that meat of cow and buffalo and skin and hair of all the animal species under study can be identified by using double gel immunodiffusion test. The use of absorbed antiserum is not specific to prevent cross-reaction occurring in camel meat antisera and its skin antigen and this needs further investigation in this respect.

4.6. Agar Gel Electrophoresis

For the characterization of meat, skin and hair antigens of cow, buffalo and camel, agar gel electrophoresis was conducted. The electrophoretic mobility of all the nine antigens was studied. Results of the electrophoretic mobility are summraized in Table 6 and 7 and are represented in Fig. 10, 11 and 12. The results recorded are as follows :-

4.6.1. Cow meat antigen

Electophoretogram of cow meat antigen revealed six protein fractions, out of which three each were present in anodic and cathodic zone [Fig. 10(1)].

4.6.2. Cow skin antigen

A total of five protein fractions of cow skin antigens were obtained on agar gel electrophoresis. Two out of five were on anodic and three were on cathodic side [Fig. 10(2)].

4.6.3. Cow hair antigen

Agar gel electrophoresis of this antigen unfolded a total of eight protein fractions, three were found to be on anodic side and five were found to be on cathodic side [Fig. 10(3)].

4.6.4. Buffalo meat antigen

This meat antigen revealed eight protein fractions in agar gel electrophoresis out of which four each were present on anodic and cathodic side [Fig. 11(4)].

Table 6 : Electrophoretic mobility of meat, skin and hair antigens of cow, buffalo and camel in agar gel electrophoresis.

Sr. No.	Type of species and antisera	Number of protein fractions in electrophoretogram		
		Anodic	Cathodic	Total
1.	Cow meat	3	3	6
2.	Cow skin	2	3	5
3.	Cow hair	3	5	8
4.	Buffalo meat	4	4	8
5.	Buffalo skin	4	3	7
6.	Buffalo hair	2	2	4
7.	Camel meat	4	3	7
8.	Camel skin	4	2	6
9.	Camel Hair	4	4	8

Table 7 : Relative percentage of meat, skin and hair antigens of cow, buffalo and camel, estimated by agar gel as electrophoresis.

Sr. No.	Type of species and antigen	Protein fractions of antigens in per cent							
		Anodic side						cathodic side	
		1st	2nd	3rd	4th	5th	6th	7th	8th
1.	Cow meat	7.22	13.74	37.45 *	27.14	8.25	6.20	-	-
2.	Cow skin	13.78	10.74 *	15.53	34.39	25.56	-	-	-
3.	Cow hair	10.45	7.59	19.97 *	18.51	27.64	4.96	4.18	6.70
4.	Buffalo meat	4.34	5.17	12.08	18.67 *	16.09	17.24	11.20	15.22
5.	Buffalo skin	6.29	11.33	5.19	9.09 *	17.07	26.42	24.61	-
6.	Buffalo hair	20.14	3.98 *	13.74	62.14	-	-	-	-
7.	Camel meat	11.37	9.90	19.08	12.99 *	16.98	19.05	10.63	-
8.	Camel skin	27.41	12.44	11.52	13.94 *	18.54	16.15	-	-
9.	Camel hair	2.72	4.99	12.71	8.48 *	6.04	5.40	38.12	21.54

Note : * denotes place of feeding of samples.

Figure 10 : Agar gel electrophoretic pattern showing the different protein fractions of cow's meat(1), skin(2) and hair(3) antigens.

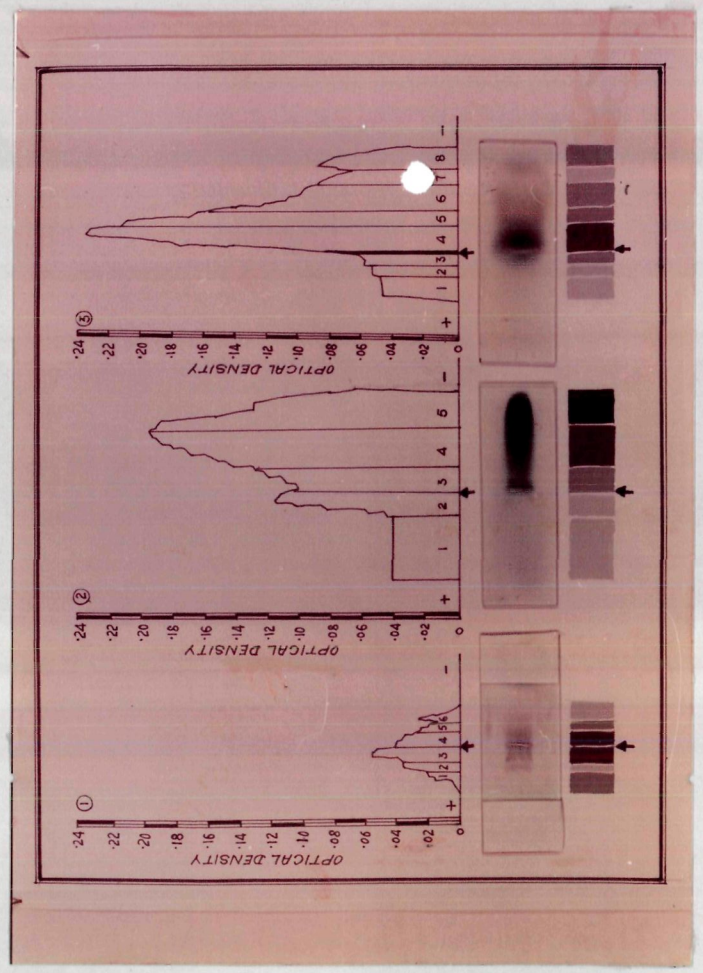
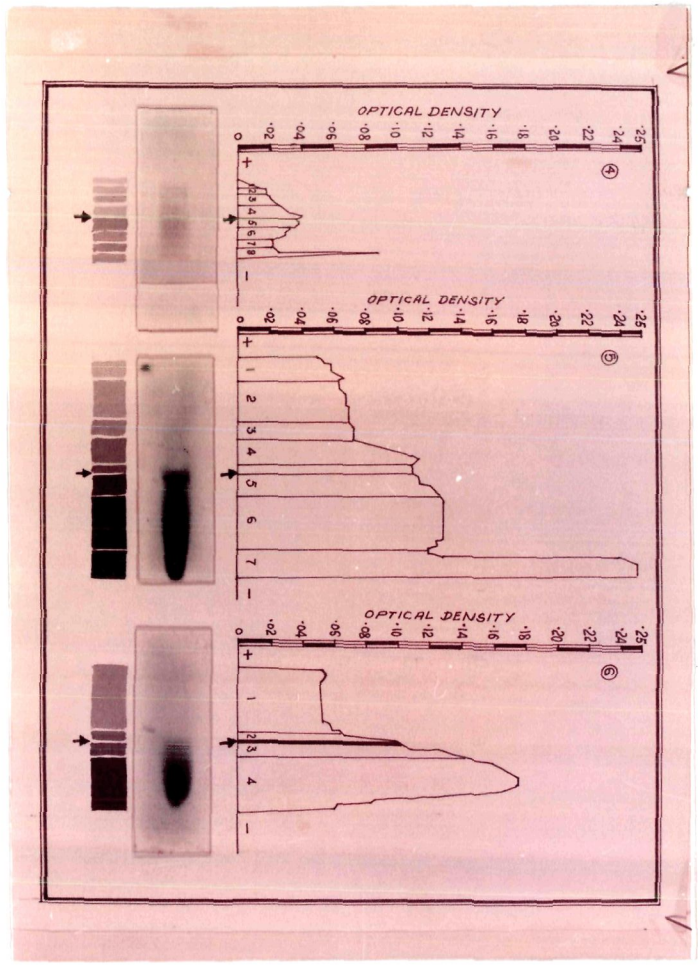

OPTICAL DENSITY
·24 ·22 ·20 ·18 ·16 ·14 ·12 ·10 ·08 ·06 ·04 ·02 0
1 2 3 4 5 6 7 8
OPTICAL DENSITY
·24 ·22 ·20 ·18 ·16 ·14 ·12 ·10 ·08 ·06 ·04 ·02 0
1 2 3 4 5 6
OPTICAL DENSITY
·24 ·22 ·20 ·18 ·16 ·14 ·12 ·10 ·08 ·06 ·04 ·02 0
1 2 3 4 5 6

Figure 11 : Agar gel electrophoretic pattern showing the different protein fractions of buffalo's meat(4), skin(5) and hair(6) antigens.


OPTICAL DENSITY
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1 2 3 4 5 6 7 8
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OPTICAL DENSITY
0 ·02 ·04 ·06 ·08 ·10 ·12 ·14 ·16 ·18 ·20 ·22 ·24 ·25
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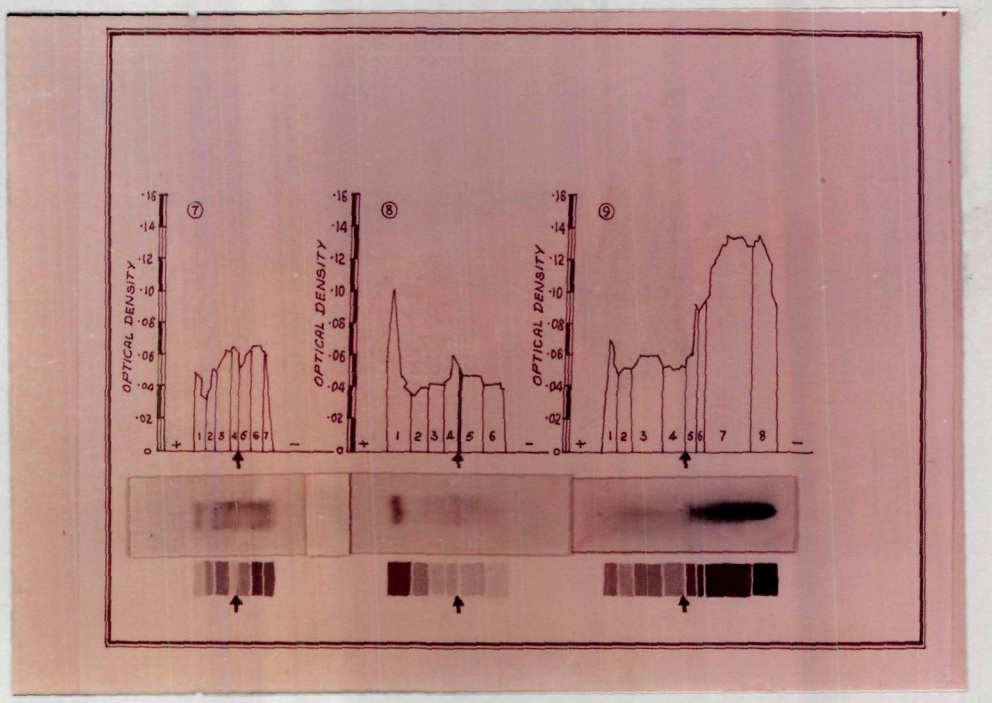
Figure 12 : Agar gel electrophoretic pattern showing the different protein fractions of camel's meat(7), skin(8) and hair(9) antigens.


⑦
⑧
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OPTICAL DENSITY
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1 2 3 4 5 6 7
1 2 3 4 5 6
1 2 3 4 5 6 7 8
–

1701533

4.6.5. Buffalo skin antigen

On agar gel electrophoresis, this antigen yielded a total of seven protein fractions and among them four were on anodic side and three were on cathodic side [Fig. 11(5)]

4.6.6. Buffalo hair antigen

On agar gel electrophoresis of the said antigen, four protein fractions were, obtained where two each fractions were seen at anodic and cathodic side [Fig. 11(6)].

4.6.7. Camel meat antigen

Seven protein fractions were unfolded on the agar gel electrophoresis of this antigen, and out of these four were on anodic side and three were on cathodic side [Fig. 12(7)].

4.6.8. Camel skin antigen

It was found that this antigen yielded six protein fractions on agar gel electrophoresis, out of which four were on anodic side and two were on cathodic side [Fig. 12(8)].

4.6.9. Camel hair antigen

Agar gel electrophoresis of this antigen revealed a total of eight protein fractions. Four fractions each were seen on anodic and cathodic side [Fig. 12(9)].

The antigens prepared from five different samples of meat, skin and hair of each species showed an identical protein pattern on agar gel electrophoresis. In the present investigation, the electrophoretic pattern showed that some of the fractions with higher percentage in all the three species under study were located near the feeding line at anodic side. The results of electrophoretic pattern of skin antigens of cow and buffalo showed some fractions with higher percentage at cathodic side while, in the case of camel the fraction with higher percentage was located at anodic side. The hair antigens of all the three species showed some fractions with highest percentage towards cathodic side.

Isoelectric focusing technique has been used by various researchers viz. Slattery and Sinclair (1983), King (1984) and Slattery et al. (1984). Sherikar et al. (1986) used this technique for identification of meat and observed eleven fractions in cow and ten fractions in buffalo meat whereas in the present study, the agar gel electrophoretic pattern yielded six and eight fractions, respectively for cow and buffalo meat antigens. The less number of fractions observed in our study is probably due to the difference in the technique. The variation in the number of protein fraction of cow, buffalo and camel meat have also been observed by Abdallah et al. (1983). The result of present work is also in line with the work of above worker where difference in protein fractions of meat of each species gives a characteristic pattern. Agar gel electrophoresis technique has also been used earlier by Jain (1993) to characterize proteins of

raw meat of nine avian species. Further, he emphasized that this technique can be used for identification of meat as it gives a characteristic persistent pattern for meat antigens of each species. Presently, our findings are also in agreement with his work.

The agar gel electrophoretic technique seems to be much easier and the results can be secured in, on an average 45 minutes. It is further helpful due to increasing quality consciousness, demand for specific meat and for enforcement of strict legislation, which is essential for quick identification of species of origin of meats.

Matoltsy and Matoltsy (1963) observed five fractions in stratum corneum in disc electrophoresis while, Lahiri *et al*. (1983) observed five fractions in skin of a fish whereas, in the present investigation 5, 7 and 6 fractions were observed, respectively in skin antigens of cow, buffalo and camel. On comparing with the work of these researchers, the difference in the protein fractions obtained in the present study is due to the change in species.

In the present study, the electrophoretic pattern of hair antigens revealed 8, 4 and 8 fractions, respectively in cow, buffalo and camel. Matoltsy and Matoltsy (1963) by using disc electrophoresis observed two fractions in human hair while, Shechter *et al*. (1969) observed 9, 14, 13, 9 and 10 fractions respectively in human, monkey, dog, guinea pig and rabbit. Simonsen (1971) observed five fractions in bovine hair. On

comparison it can be concluded that hair antigen of each species showed difference in number of fractions. Results of the present study showed eight fractions in cow hair and four in buffalo hair which are not in agreement with the work of Simonsen (1971). This may be probably due to the difference in the technique used during present investigation. However, three to four fractions were observed in acrylamide gel electrophoresis of sheep wool by Purohit *et al*. (1983) which is quite close to the number of fractions observed in buffalo hair in the present study. It can be concluded that though the number of fractions is same but each fraction show difference in protein percentage and on processing of five samples of each antigen of the three species, a consistent finding about the trend of fractions was noticed.

Results of the present study can be helpful in vetero-legal cases as hairs are not damaged for a pretty long time in comparison to meat and skin and the sample can be processed without any dispute as there is no question of putrefaction. The advantage of agar gel electrophoresis test for characterization of antigens is that, it needs less time to characterize antigenic protein. The test is simple, rapid and a permanent proof in the form of stained slide for record is also available.

4.7. Immunoelectrophoresis

For the characterization of antigens of meat, skin and hair of cow, buffalo and camel, immuno electrophoresis technique was applied. All the antigens and their homologous

antisera raised in rabbits, gave a variable number of precipitin arcs for each antigen in immunoelectrophoresis. The results are summarized in Table 8 and are represented in Fig. 13 to 21. The results so observed are described as under.

4.7.1. Cow meat antigen with its antiserum

After electrophoresis of cow meat antigen, when it was allowed to react with its homologous antiserum, it revealed three precipitin arcs. Two were located in cathodic zone and one in anodic zone (Fig. 13).

4.7.2. Cow skin antigen with its antiserum

After electrophoresis of cow skin antigen, when it was allowed to react with its antiserum, it showed three precipitin arcs. Two were located in cathodic zone and one in anodic zone (Fig. 14).

4.7.3. Cow hair antigen with its antiserum

Cow hair antigen was electrophoresed and allowed to react with its homologous antiserum. Two precipitin arcs were found which were located in cathodic zone (Fig. 16).

4.7.4. Buffalo Meat antigen with its antiserum

After electrophoresis of buffalo meat antigen, when it was allowed to react with its homologous antiserum, it revealed nine precipitin arcs, out of which one was in anodic zone, two in common zone of anode and cathode and six in cathodic zone (Fig. 16).

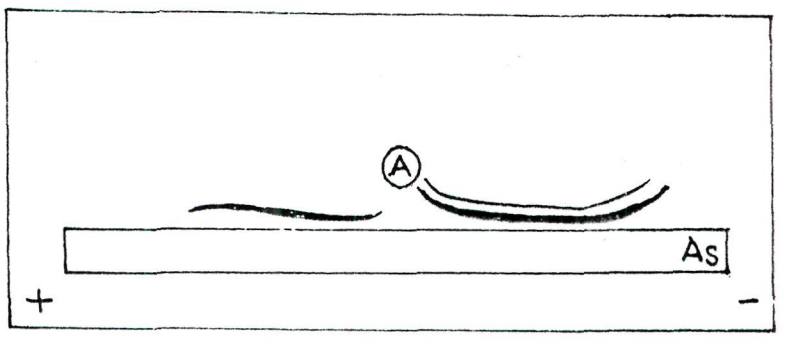
Table 8 : Distribution of precipitin arcs as observed in immunoelectrophoretic reactions of various antigens of cow, buffalo and camel with their respective antiserum.

Sr. No.	Type of species and antisera	Number of precipitin arcs located in anodic zone	cathodic zone	between both zones	Total
1.	Cow meat	1	2	–	3
2.	Cow skin	1	2	–	3
3.	Cow hair	–	2	–	2
4.	Buffalo meat	1	6	2	9
5.	Buffalo skin	1	1	1	3
6.	Buffalo hair	–	–	1	1
7.	Camel meat	6	6	1	13
8.	Camel skin	3	–	–	3
9.	Camel Hair	1	1	–	2

Figure 13 : Photograph (a) and schematic diagram (b) showing immunoelectrophoretic pattern of cow meat antigen(A) and rabbit anti-cow meat serum (As) (unstained slides).

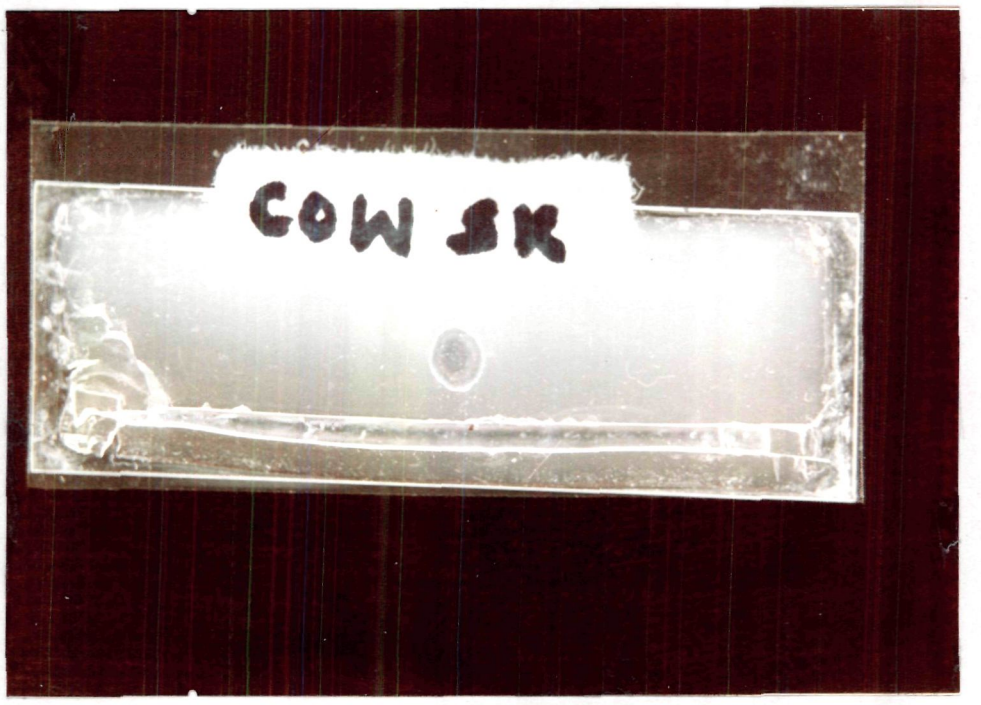
(a)

COW MEAT



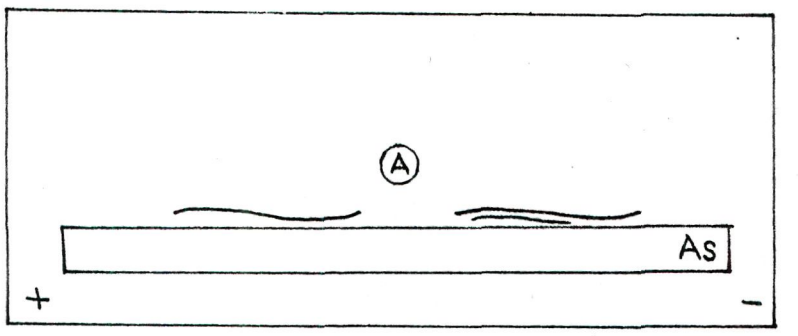
(b)

Figure 14 : Photograph (a) and schematic diagram (b) showing immunoelectrophoretic pattern of cow skin antigen(A) and rabbit anti-cow skin serum (As) (unstained slides).



(a)

COW SKIN



(b)

Figure 15 : Schematic diagram showing immunoelectrophoretic pattern of cow hair antigen(A) and rabbit anti-cow hair serum (As).

COW HAIR

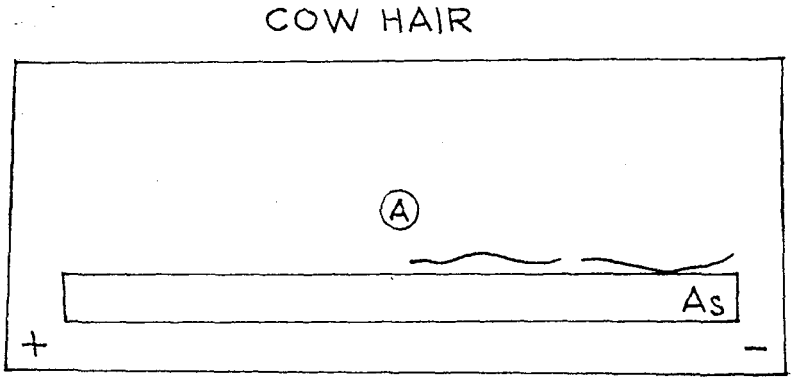
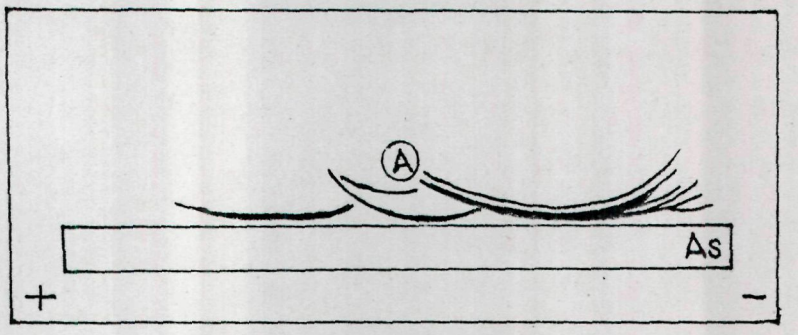


Figure 16 : Photograph (a) and schematic diagram (b) showing immunoelectrophoretic pattern of buffalo meat antigen(A) and rabbit anti-buffalo meat serum (As) (unstained slides).

(a).

BUFFALO MEAT

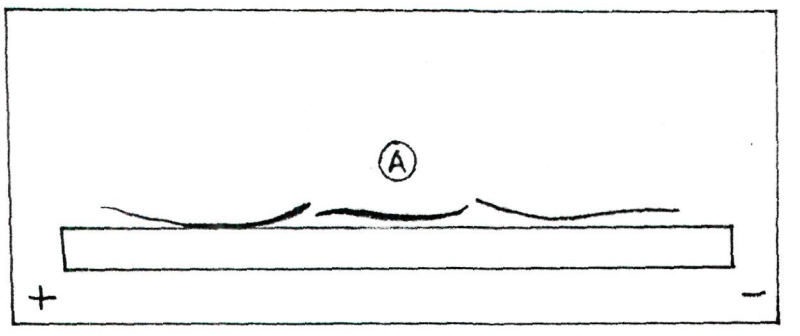


(b)

Figure 17 : Photograph (a) and schematic diagram (b) showing immunoelectrophoretic pattern of buffalo skin antigen(A) and rabbit anti-buffalo skin serum (As) (unstained slides).

(a)

BUFFALO SKIN



(b)

Figure 18 : Schematic diagram showing immunoelectrophoretic pattern of buffalo hair antigen(A) and rabbit anti-buffalo hair serum (As).

BUFFALO HAIR

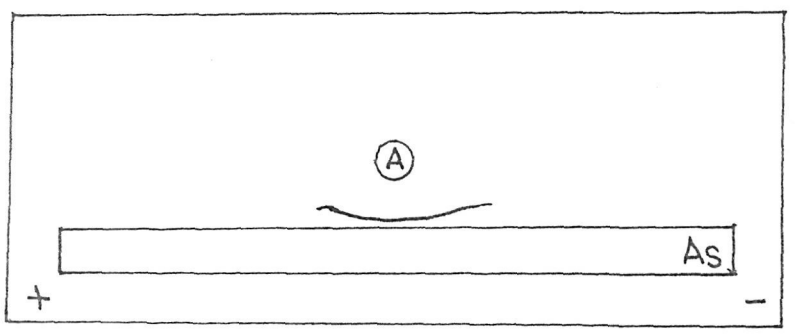
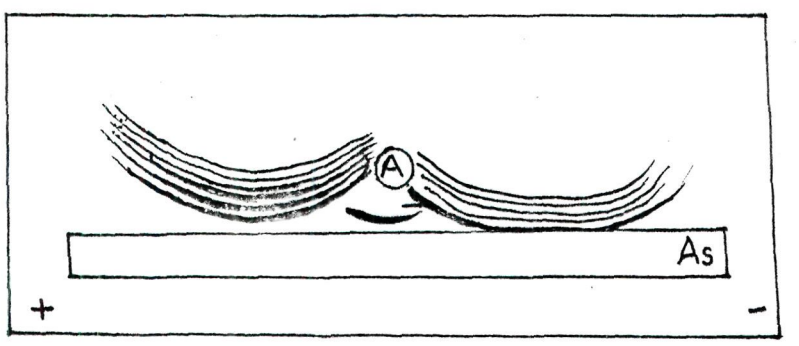


Figure 19 : Photograph (a) and schematic diagram (b) showing immunoelectrophoretic pattern of camel meat antigen(A) and rabbit anti-camel meat serum (As) (unstained slides)

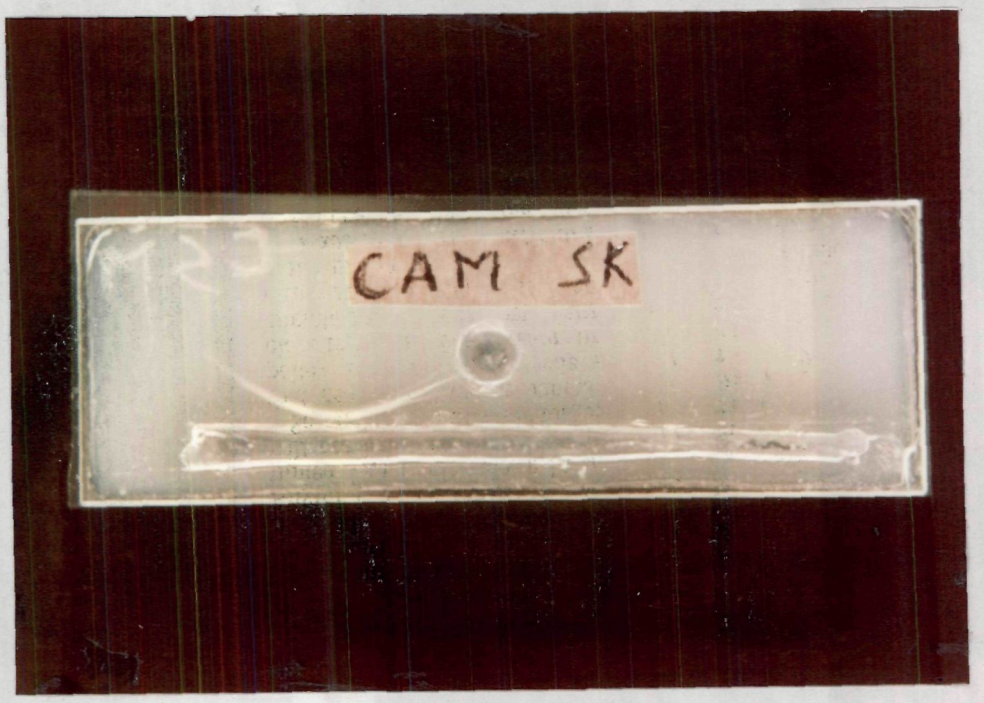
(a)

CAMEL MEAT



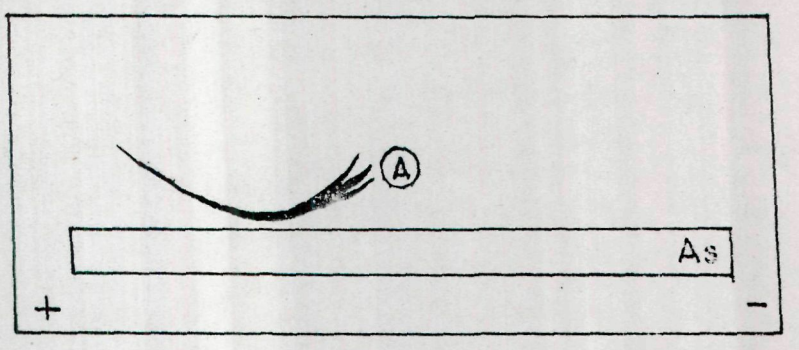
(b)

Figure 20 : Photograph (a) and schematic diagram (b) showing immunoelectrophoretic pattern of camel skin antigen(A) and rabbit anti-camel skin serum (As) (unstained slides).



(a)

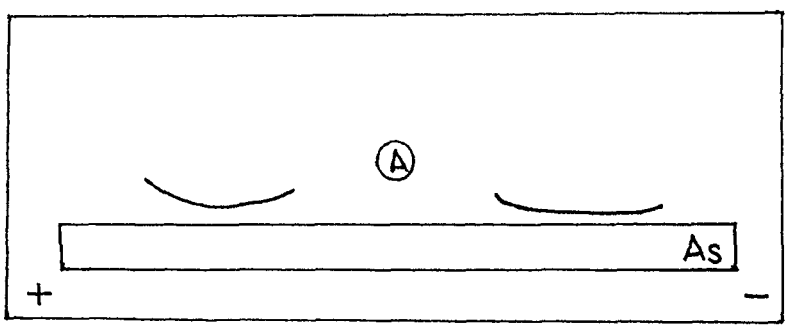
CAMEL SKIN



(b)

Figure 21 : Schematic diagram showing immunoelectrophoretic pattern of camel hair antigen(A) and rabbit anti-camel hair serum (As).

CAMEL HAIR



4.7.5. Buffalo skin antigen with its antiserum

The skin antigen of buffalo was electrophoresed and when it reacted with its homologous antiserum, it revealed a total of three precipitin arcs. One arc each was present in anodic zone, common zone of anode and cathode and in cathodic zone, respectively (Fig. 17).

4.7.6. Buffalo hair antigen with its antiserum

The buffalo hair antigen after electrophoresis was allowed to react with its homologous antiserum and it revealed only one precipitin arc which was common to both anodic and cathodic zone (Fig. 18).

4.7.7. Camel meat antigen with its antiserum

After electrophoresis of the antigen, when it was allowed to react with its homologous antiserum, it revealed a total of thirteen precipitin arcs. Six arcs each were located in anodic and cathodic zone. One arc was common to both the zones (Fig. 19).

4.7.8. Camel skin antigen with its antiserum

When the skin antigen of camel was electrophoresed and allowed to react with its homologous antiserum, it revealed three precipitin arcs which were in the anodic zone (Fig. 20).

4.7.9. Camel hair antigen with its antiserum

When the hair antigen of camel was electrophoresed and allowed to react with its homologous antiserum, it revealed two precipitin arcs in which one each was located in the cathodic and anodic zone (Fig. 21).

Immunoelectrophoresis is a technique for the study of antigens and antibodies. It is based on two of their properties of ability to precipitate in agar gel with specific antibody and antigen (immunodiffusion), and characteristic mobility in an electric field (electrophoresis).

At the end of electrophoretic separation, each protein had moved through the agar gel to a particular distance, characteristic to its molecular weight. Arcs of antigen-antibody precipitate resulted one for each protein antigen present in the original solution.

The results of electrophoretic migration were rapid and without undue heating of electrophoretic medium and gel distortion, when barbitone buffer with pH 8.6 and 0.025 M ionic strength was used. This finding is in accordance with the work of Bennet and Boursnell (1962) and Ornsttein (1964).

Ramadass and Misra (1981) in their studies over meat, concluded that nature and position of precipitin arcs were specific for each species and same type of characteristic findings are obtained for meat antigens in the present study.

It is evident from the present study that meat extract yielded 3 to 13 principal immunologically and serologically active antigen having different charges and mobility in electric field and these showed specific pattern to recognize the individual species of cow, buffalo and camel. Cow meat showed lowest, while camel meat showed the highest number of precipitation arcs.

Bubloz (1962) observed single precipitin line in beef and horse flesh, while Bakshi (1981) observed one anodic and four cathodic precipitin lines each for camel and buffalo, respectively. In contrast to the work of Bakshi (1981) one anodic precipitin line was also seen in the meat of buffalo but our results disagree with his findings in case of the number of cathodic lines as more number of cathodic lines in buffalo and camel were observed in the present study.

The nature and position of precipitin arc as observed in immunoelectrophoresis in the present study reveals that, there is a definite pattern to recognize meat antigen of species, which has also been reported earlier by Sinell and Mentz (1969), Ramadass and Misra (1981) for characterization of ruminant meat antigens and recently it has also been recommended by Jain (1993), who conducted the similar study to identify the meat of nine avian species.

The present observation revealed three precipitin lines for cow, buffalo and camel skin, but there was sharp differentiation in location and identity of each arcs. Such

results can be used to identify the skin of these species. It is worthy to note that camel skin showed three precipitin arcs only at anodic zone in comparison to the results of cow and buffalo skin. The references regarding the discussion in this aspect could not be traced out, but the results of this investigation will definitely be helpful in solving out the disputed cases.

As evident from the present work, the hair antigens showed a minimum number of precipitation arcs in comparison to the meat and skin antigens of the species under study. Cow and camel hair antigen showed two, while buffalo hair antigen showed only one precipitation arc. Further, on comparing the results of all the three species, the hair antigen and antibody after reaction showed sharp differences in the location of precipitin arcs. Heterogenecity in the hair antigens of man, cattle, horse, sheep, goat, pig, cat and dog was also observed during immunoelectrophoretic studies by Simonsen (1976) and the results of present investigation for hair antigens of all the three species yielded similar trends of heterogenecity.

Electrophoretic separation followed by double gel immunodiffusion provides a higher degree of resolving power. A more number of precipitin arcs may be due to the result of better separation of antigenic components under electric field. Presentely this technique has proved usefulness for detecting and identifying individual component in a multiple component system.

Though, our observations on serological identification of skin and hair is preliminary, but this study will definitely pave way for its application in the field of disputed cases where only skin and hair samples are submitted for the identification of species. Further, the result of the present study may be utilized in checking adulteration of meat supplied for human consumption. The evidences made by the tests so applied will certainly be helpful in our court of law for apprehension of unlawful persons and will provide safeguard to animals which are rare or whose slaughter is banned. Also, it will be helpful to safeguard the religious sentiments of the various communities.

SUMMARY

5. SUMMARY

Increased demand of animal proteins in the form of meat in human diet has created unlawful temptation of slaughtering animals whose slaughter is banned in India or whose meat consumption is not allowed by the authorities. The present investigation on animal species identification was attempted to help the court of law by complimenting evidence with support.

For characterization of meat, skin and hair, antigens were prepared by using freezing and thawing technique. The total protein contents were 0.89, 0.75, 0.57; 1.39, 1.13, 0.67; 1.27, 1.06 and 0.96 gram per cent for meat, skin and hair antigens of cow, buffalo and camel, respectively.

Antiserum against each antigen was raised in rabbits by inoculating antigens with Freund's incomplete adjuvant and antibiotic (Gentamicin). The immunization schedule followed for raising antiserum against various antigens was 0, 3rd, 6th and 16th day. A total of 40mg protein in split doses of 10mg per inoculation by subcutaneous route was given in each rabbit at their flank region. Blood was collected after 4 days of the last inoculation through cardiac puncture and serum was separated by centrifugation. The antiserum and antigens were stored at -20^{o} C in deep freezer after adding thiomersal of 1:5000 concentration as preservative.

Double gel immunodiffusion test was applied to unabsorbed antiserum and antigens using homologous antigen-antibody system. The result showed 4, 3, 1; 4, 3, 1; 7, 2 and 2

precipitation bands with antigens and unabsorbed antiserum of meat, skin and hair of cow, buffalo and camel, respectively.

To determine antigenic correlation among various antigens of animal species under study, cross-reaction tests were performed by using double gel immunodiffusion technique. For this, unabsorbed antisera was allowed to react with its homologous and eight heterologous antigens. Cross-reactions were observed in majority of antigens but none of antisera cross-reacted with all eight heterologous antigens. Antisera of buffalo hair, camel skin and hair did not show cross-reaction with any of the heterologous antigens. The meat antiserum of cow, buffalo and camel showed a common precipitation reaction with their own meat as well as their skin antigen.

Double gel immunodiffusion test was applied by using absorbed antisera with homologous and heterologous antigens. Except camel meat, the test revealed species-specific reaction with homologous antigens. Camel meat antiserum was absorbed with eight other antigens but even then it cross-reacted with camel skin antigen. With absorbed antiserum weak reactions with few precipitation lines were observed in this test.

For characterization of various antigens of animal species, agar gel electrophoresis was carried out by passing a current of 50mA with potential difference of 120 volts for 35 to 50 minutes. For each antigen, a characteristic pattern of protein fraction was observed with marked anodic and cathodic mobility. The number of protein fractions or bands observed with

meat, skin and hair antigens of cow, buffalo and camel were 6, 5, 8; 8, 7, 4; 7, 6 and 8, respectively.

The electrophoretograms of each antigen were put into densitometer (SYSTRONICS-201) and their optical densities were measured. Relative percentage of protein fractions of each antigen were estimated by plotting the graph by taking optical density on ordinate and various fractions on abscissa.

In immunoelectrophoresis, antigenic fractions of each antigen under study were separated in agar gel medium under electric field and then they were reacted with homologous antiserum. A definite number of precipitin arcs were observed for each antigen of animal species. A total of 3, 3, 2; 9, 3, 1; 13, 3 and 2 precipitin arcs were recorded for meat, skin and hair antigens of cow, buffalo and camel, respectively. Except cow and buffalo hair antigen, precipitin arcs were observed in both anodic and cathodic zones. In the reaction of cow hair antigen, precipitin arcs were present in cathodic zone and in antigen of buffalo hair the single precipitin arc was observed in a zone which was common to anodic and cathodic side. In immunoelectrophoresis, more number of precipitin arcs may be due to the result of better separation of antigenic components of the antigens used in this study.

The result of the present investigation will help in checking malpractices in meat industry and unlawful slaughter of animals so as to safeguard the religious sentiments of the consumers.

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SEROLOGICAL CHARACTERIZATION OF MEAT, SKIN AND HAIR OF COW, BUFFALO AND CAMEL

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ABSTRACT

The present serological study was undertaken to characterize meat, skin and hair antigens of cow, buffalo and camel. The meat, skin and hair and antigens were prepared by freezing and thawing technique. For raising antiserum against all the antigens, the rabbits were inoculated with antigens on 0, 3rd, 6th and 16th day by subcutaneous route. Antigens were inoculated at the rate of 40mg of total protein in 10mg split doses.

The antisera thus obtained was used as a source of antibodies against desired antigen in serological reactions viz. double gel immunodiffusion and immunoelectrophoresis tests. Antigens were also processed by agar gel electrophoresis for characterization of their antigenic components.

Double gel immunodiffusion test was conducted by reacting unabsorbed antisera with its homologous antigen and it showed 4, 3, 1; 4, 3, 1; 7, 2 and 2 precipitation bands in meat, skin and hair antigens of cow, buffalo and camel, respectively.

To determine the antigenic correlation among the various antigens of animal species under study, the unabsorbed antiserum was reacted with its homologous and eight heterologous antigens. Cross-reactions were observed in majority of antigens but none of antisera cross-reacted with all the eight antigens. Buffalo hair, camel skin and hair antiserum did not show any cross-reaction. The meat antisera of cow, buffalo and camel showed a common precipitation reaction with their meat as well as the skin antigens.

The absorbed antiserum was used in double gel immunodiffusion test to demonstrate species-specific reaction. Except camel meat, the test revealed species-specific weak reaction with reduced number of precipitin lines. The absorbed camel meat antisera also cross-reacted with camel skin antigen.

All the antigens were characterized by using agar gel electrophoresis technique. A characteristic pattern of protein fractions was obtained for each antigen. The number of protein fractions observed were 6, 5, 8; 8, 7, 4; 7, 6 and 8 for meat, skin and hair antigens of cow, buffalo and camel, respectively and their protein percentage was also determined.

In immunoelectrophoresis the antigenic fraction were separated out and then these were allowed to react with their respective homologous antiserum. A definite number of precipitin arcs were observed for each antigens i.e. 3, 3, 2; 9, 3, 1; 13, 3 and 2 for antigens of meat, skin and hair of cow, buffalo and camel, respectively. Precipitation arcs were present both in anodic and cathodic zones except in the case of cow and buffalo hair antigens. In the reaction of cow hair antigen the precipitin arcs were present only in cathodic zone while in case of buffalo hair antigen the precipitin arc was common to both the anodic and cathodic zone.

The results of the present study will help in checking malpractices of unlawful slaughter of animals and adulteration of meat meant for human consumption and will also safeguard the religious sentiments of the various communities.

गाय, भैंस व ऊंट के आमिष, चर्म व बालों का लासिकीय अभिज्ञान

स्नातकोत्तर शोध-ग्रन्थ

पशु चिकित्सकीय जन स्वास्थ्य,
औषध विभाग,
पशु चिकित्सा एवं पशु विज्ञान महाविद्यालय,
राजस्थान कृषि विश्वविद्यालय, बीकानेर ।

प्रस्तुतकर्ता : **शुभेन्दु दीक्षित**

- : अनुक्षेपण : -

गाय, भैंस व ऊंट के आमिष, चर्म व बालों के लासिकीय पहचान के लिए यह अध्ययन किया गया । आमिष, चर्म एवं बालों का एंटीजन अवशीतन व पुनः पिघलाने की प्रणाली से तैयार किया गया । प्रत्येक खरगोश में ०, ३, ६ एवं १६ वें दिवस के अन्तराल पर कुल ४० मि.ग्राम प्रोटीन को १० मिली ग्राम की विभाजित मात्रा में सबक्युटेनियसली लगाकर प्रत्येक एंटीजन के विरूद्ध एंटीसीरम तैयार की गई ।

इस प्रकार प्राप्त एंटीसीरम का लासिकीय प्रतिक्रियाओं, जैसे 'डबल जेल इम्युनोडिफ्युजन' एवं 'इम्युनोइलेक्ट्रोफोरेसिस' परीक्षणों में एंटीजन के विरूद्ध एंटीबाडी स्त्रोत के रूप में उपयोग किया गया । अध्ययन के अन्तर्गत प्रत्येक एंजीटन के एंटीजैनिक अवयवों के लाक्षणिक वर्णन के लिए एंटीजनों को 'अगार जेल एलेक्ट्रोफोरेसिस' से संसाधित किया गया ।

अनावशोषित (अनएब्जोर्व्ड) एंटीसीरम को सजातीय एंटीजन से क्रिया कराके डबल जेल इम्युनोडिफ्युजन परीक्षण किया गया और इस प्रक्रिया द्वारा ४, ३, १; ४, ३, १; ७, २ एवं २ प्रेसिपिटेशन बेन्डस क्रमशः गाय, भैंस व ऊंट के आमिष, चर्म एवं बालों में पाये गये ।

अध्ययन के अन्तर्गत विभिन्न एंटीजनों में एंटीजैनिक सह-सम्बन्ध (कोरिलेशन) निर्धारित करने के लिए अनावशोषित एंटीसीरम की तत्सजातीय (होमोलोगस) एवं आठ विजातीय (हेटरोलोगस) एंटीजनों के साथ डबल जेल इम्युनोडिफ्युजन परीक्षण में प्रतिक्रिया करवाई गई । भैंस के बाल, ऊंट की चर्म और बाल के एंटीसीरम के अलावा अन्य एंटीसीरम में क्रास-रिएक्शन होना पाया गया, लेकिन किसी भी एंटीसीरम ने समस्त विजातीय एंटीजनों के साथ क्रास-रिएक्शन प्रदर्शित नहीं किया । गाय, भैंस व ऊंट के आमिष के एंटीसीरम ने स्वयं के आमिष के एंटीजनकेअलावा स्वयं के चर्म के एंटीजन के साथ भी प्रतिक्रिया प्रदर्शित की ।

प्रजाति-विशिष्ट क्रिया निरूपण करने के लिए अवशोषित (एब्जोर्ब्ड) एंटीसीरम को डबल जेल इम्युनोडिफ्युजन परीक्षण में प्रयुक्त किया गया । ऊंट के आमिष के एंटीसीरम के अलावा सभी अवशोषित एंटीसीरम ने केवल सजातीय एंटीजन से ही प्रतिक्रिया दर्शाई । इस परीक्षण में पूर्व के परीक्षण की तुलना में धुंधले व कम संख्या में प्रेसिपिटेशन बेन्ड प्राप्त हुए । ऊंट के आमिष की अवशोषित एंटीसीरम ने स्वयं के आमिष के एंटीजन के अलावा अपने ही चर्म के एंटीजन के साथ भी प्रतिक्रिया की ।

समस्त एंटीजनों का अगार जेल इलेक्ट्रोफोरेसिस तकनीक को प्रयोग में लाते हुए लाक्षणिक चित्रण किया गया एवं अभिलाक्षणिक प्रेसिपिटेशन बेन्ड पैटर्न प्राप्त किये गये । इसके ६, ५, ८; ८, ७, ४; ७, ६ एवं ८ प्रोटीन अंश क्रमशः गाय, भैंस व ऊंट के आमिष, चर्म एवं बालों के एंटीजनों में पाये गये एवं सभी एंटीजनों के प्रत्येक अंश का प्रोटीन प्रतिशून्य भी ज्ञात किया गया ।

इम्युनोइलेक्ट्रोफोरेसिस परीक्षण के लिए सभी एंटीजनों के एंटीजैनिक अवयवों को पहले पृथक किया गया और फिर उनकी सजातीय एंटीसीरम के साथ प्रतिक्रिया कराई गयी । प्रत्येक एंटीजन के लिए एक निश्चित संख्या में प्रेसिपिटीन आर्क अवलोकित हुए । गाय, भैंस व ऊंट के आमिष, चर्म एवं बालों में क्रमशः ३, ३, २; ९, ३, १; १३, ३ एवं २ प्रेसिपिटेशन आर्क दिखाई दिये । इस परीक्षण में गाय व भैंस के बालों के अलावा सभी एंटीजनों में प्रेसिपिटेशन आर्क एनोडिक एवं केथोडिक क्षेत्रों में पाये गये । गाय के बालों की प्रतिक्रिया में समस्त प्रेसिपिटेशन आर्क केथोडिक क्षैत्र में तथा भैंस के बालों की प्रतिक्रिया में एकमात्र प्रेसिपिटेशन आर्क एनोड और केथोड क्षेत्र के बीच में पाया गया ।

इस अध्ययन के परिणामों से पशुओं के अवैध वध एवं खाद्य के रूप में काम लिए जाने वाले आमिष में होने वाली मिलावट पर रोक लगाने में तथा विभिन्न समुदायों की धार्मिक भावनाओं को बनाये रखने में मदद मिलेगी ।

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APPENDIX

APPENDIX - I

Composition and preparation of buffers and reagents used

I LOWRY'S REAGENTS

(i)	Sodium hydroxide solution (5N)	
	Sodium hydroxide flakes	200 g
	Distilled water	1000 ml
(ii)	Standard tyrosine solution (0.02 per cent)	
	Tyrosine	0.2 g
	Hydrochloric acid (0.1N)	1000 ml
(iii)	Folin-Ciocalteu reagent	
	Sodium tungstate (BDH)	100 g
	Sodium molybdate (BDH)	25 g
	Phosphoric acid (85 per cent)	50 ml
	Hydrochloric acid (concentrated)	100 ml
	Lithium sulphate	150 g
	Liquid bromine	few drops
	Glass distilled water to make volume	1000 ml
(iv)	Tyrosine standard	
	Standard tyrosine solution (0.02 per cent)	4 ml
	Sodium hydroxide solution (5N)	2 ml
	Folin-Ciocalteu reagent	3 ml
	Glass distilled water to make volume	1000 ml
(v)	Blank solution	
	Sodium hydroxide solution (5N)	2 ml
	Folin-Ciocalteu reagent	3 ml
	Glass distilled water to make volume	50 ml

II PHOSPHATE BUFFER (0.028M) pH 7.5

Sodium dihydrogen phosphate	0.156 g
Disodium hydrogen phosphate	1.596 g
Glass distilled water to make volume	1000 ml

III VERONAL BUFFER (0.05M) pH 8.6

Barbituric acid	1.84 g
Sodium diethyl barbiturate	10.30 g
Glass distilled water to make volume	1000 ml

IV PHOSPHATE BUFFER SALINE (0.01M) pH 7.5

Sodium dihydrogen phosphate	0.380 g
Disodium hydrogen phosphate	1.020 g
Sodium chloride	0.800 g
Glass distilled water	100 ml

V AGAR GEL MEDIUM (1.5 PER CENT)

(i) For immunodiffusion :

Noble agar powder (Difco)	1.5 g
Phosphate buffer pH 7.5	50 ml
Glass distilled water	50 ml

(ii) For electrophoresis

Noble agar powder (Difco)	1.5 g
Veronal buffer pH 8.6	50 ml
Glass distilled water	50 ml

Dissolved by heating over the flame and autoclaved at 10 pounds pressure for 10 minutes. The molten agar was allowed to cool at 50 ^{o}C and thiomersal was added to a final concentration of 0.02 per cent as preservative.

VI AMIDO BLACK STAIN (0.1 PER CENT)

Amido black dye	1 g
Glacial acetic acid (1M)	425 ml
Sodium acetate (0.1M)	425 ml
Glycerol	150 ml

VII DESTAINING SOLUTION

Glacial acetic acid	2 ml
Glycerol	10 ml
Distilled water to make volume	100 ml

Note : 1. The desired pH of the buffers was adjusted with the help of Systronics digital pH meter-335 and thiomersal was added to a final concentration of 0.02 per cent as preservative.

2. All the solutions were prepared in double glass distilled water and the chemicals used were of analar grade and stored at refrigeration temperature. The chemicals and reagents were used within one month.