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OPTIMIZATION OF COUNTERCURRENT IMMUNOELECTROPHORESIS AND AGAR GEL IMMUNODIFFUSION TESTS FOR THE COMPARATIVE DETECTION OF HORSE AND DONKEY MEAT

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ABSTRACT

Meat is the rich source of many essential amino acids (valine, lysine, methionine, leucine, tryptophan, isoleucine, phenylalanine and threonine), vitamins (Vitamin D, B6 and B12) and a very good source of nutrients. Meat is also a good source of iron that is very necessary for human body and helps in maintenance of health. The major issue in meat market now a days is the adulteration of meat with other cheap meat and unhealthy items. To avoid this adulteration in meat, authenticity has become necessary. There are many techniques for the detection of adulteration in meat but, the best techniques to detect meat protein in raw as well as in cooked meat are Agarose Gel Immunodiffusion (AGID) and Countercurrent Immunoelectrophoresis (CCIE). Meat extracts (antigens) are prepared from horse and donkey meat samples and rising of hyper immune sera is done against these antigens in rabbits. 0ut of 20 samples, 18 samples are positive that indicate equine species have similar meat proteins. For cross reactivity, both antisera show small precipitation line with antigens (horse meat extract and donkey meat extract) and antiserum against donkey meat protein give clear results. CCIE is faster, cheaper, and sensitive technique than AGID.

KEYWORDS

Agarose gel immunodiffusion, countercurrent immunoelectrophoresis, meat extracts, antisera, precipitation.

1. INTRODUCTION

Meat is composed of water, fat and proteins including amino acids like tryptophan, methionine, leucine, valine, isoleucine, phenylalanine, lysine and threonine also containing micronutrients [1]. Meat is the source of vitamins such as vitamin B6, vitamin D and vitamin B12 and contain sufficient amount of fats like polyunsaturated omega-3. It is the source of some essential supplements like zinc, selenium and zinc which are the basic requirements of human body [2].

One way it is the major source of protein and also rich in many micronutrients which Nutrients are very much beneficial for human health like iron, manganese and zinc. Normally red meat. Products like cold cut, hamburgers and sausages are cheaper as compared to red raw meat like of sheep ad beef. Reason behind this is the presence of lower contents of the costly meat in these items that's why people preferred these meat products over pure red meat. fact that these meat items can partially fulfill the nutritional requirements of the people [3,4].

Accurate monitoring of the meat origin in the meat products is required to increase the awareness among the consumers as some commercial frauds includes the incorrect labeling of the ingredients used in meat products [5]. Various techniques has been developed to for accurate supervision of these ingredients and to analyze the food products, these analytical methods are based on DNA and protein analysis [5]. Protein-based methods consists of chromatography, electrophoresis and immunological techniques [5-7]. For the maintenance and nutrition of human health the meat products are very much essential. For the development of health and proper growth in children, red meat is very important as it is rich in essential nutrients. It is also the good source of iron that is absorbed by the body in a proper way [8].

The important issues facing meat industry is the detection of adulteration and authenticity of meat. Mixing of other meat and unhealthy substances with the meat is known as adulteration. Due to mixing and adulteration of meat in meat industry, meat trading is facing unfair competition. These days, the consumers are concerned about the quality of meat and meat products they are using and conscious about choosing high quality meat products [8].

Furthermore, authenticity of meat and its detection of meat products are important issues in food regulatory control for the purpose of fraudulent replacement of higher commercial valued meat by inferior, cheaper or undesirable alternatives, the presence of undeclared species, and replacement of animal meat by plant proteins, accurate food labelling and for the evaluation of food composition and providing consumer needed information to achieve food safety [9,10].

Meat Products screening to monitor impurity and mixing is done due to many reasons behind it like ethical and economical point of view and to ensure hygiene. Economical point has gained more importance while marketing these products. To resolve these issue different tests has been designed for the evaluation of these meat products and for the detection of protein resent in meat. In these meat products quality changes occurs with the addition of components replacing quality meat with the cheap meat and also the purpose is to increase the weight of these products [11]. In retail market the biggest problem is the adulteration in ground meat. Identification of the species origin in meat samples is relevant to consumers for several reasons; the first is the possible economic loss from

fraudulent substitutions or adulterations, the second is the medical requirements of individuals who might have specific allergies, and the third is the religious reasons [12].

Islamic law forbids Muslim community from utilizing or consuming the product derived from pig meat so the consumption of Halal meat is the major issue for Musims [13]. The main authenticity issue which commonly arises among Muslim consumers is to determine whether meat products from halal species have not been mixed with material from a cheaper non-halal species. This is because in most countries, food manufacturers choose to substitute pork derivatives in food products since they are cheap and readily available [1].

In Islamic religion, meat sources of pig, donkey and horse are Haram to consume for Muslims. So it is very important for the food control laboratories to do meat differentiation of raw materials before utilization of that meat for the purpose of industrial meat products preparation and identification of animal species in these meat products [14]. According to the religious and health point of view identification and reorganization of animal species is very important. Food labeling is done to inform the consumers properly about species of meat products. According to health point of view, allergic reaction can be induced by undeclared meat proteins in immunocompromised individuals [15]. Consumption of chicken has been increasing because it has low cholesterol and fat contents than the mammalian meat. There are different techniques including isoelectric focusing, PCR (polymerase chain reaction), electrophoresis, DNA hybridization, ELISA (enzyme linked immuno sorbent assay) and chromatography used for meat detection [15].

There are many techniques, that can be used for identification of meat species, including immunochemical, serological, molecular, biological methods, and histological techniques [16-18]. Raw meat species can be identified by enzyme linked immuno sorbent assay and electrophoretic methods are simple as compared to other techniques [19].

Agarose gel Immunodiffusion test is included in immunochemical methods. It is used for the identification of specific proteins of meat species. This method involves the diffusion of antibody and antigen in agarose gel (semisolid medium) that is resulting in reaction of precipitation. Whenever, the antigen and antibody are in same concentration, the precipitin bands form on the agarose gel [20].

In meat products, identification of species-specific meat proteins by countercurrent immunoelectrophoresis (CCIE) can be done. Due to this method, the identification of different meats in raw and heat processed meat products can be done but at concentrations below 1.5%. By using species specific antibodies that are produced by raising of hyper immune sera in rabbits with heat stable antigens that is extracted from fat free meat heated at different range of temperature (75°C, 100°C for 30 min). This method was used to explain composition of declared meat products, adulteration in samples was found [21].

2. MATERIALS AND METHODS

2.1 Solutions

1. 0.05 M phosphate-buffered saline (PBS) pH 7.2
2. Tris-succinic acid pH 7.2 (0.1 M tris with 0.1 M succinic acid)
3. 50 Mm Borate buffer pH 8.2 (50M Sodium tetraborate with 50mM boric acid)
4. 0.85% Normal saline
5. Agarose
6. Ethidium bromide

2.2 Antigen preparation

According to a study, antigens for immunization were prepared from donkey and horse meat samples [21]. Homogenization of samples was done in phosphate-buffered saline (PBS) pH 7.2. Then homogenates were autoclaved and after autoclaving gauze filtration was done. Centrifugation was done at 6000 rpm for 15 mins. The supernatant were used for antibody production in rabbits.

2.3 Protein determination

Protein determination was done by proteinometer. Protein concentration was adjusted to 2mg/ml by making dilutions. Then antigen injected to rabbits with adjuvants.

2.4 Experimental animals

Local breed rabbits were purchased from Faisalabad market. They were left for a week for adaptation. 3 rabbits were used for each type of antigen.

2.5 Immunization

First dose was injected with freund's adjuvant (complete) and other doses were injected with montanide adjuvant (incomplete). 0.1ml of the antigen was inoculated subcutaneously in the rabbit on the first day and then increased daily as given in table 1.

Table 1: Schedule for immunization

Day no.	Injection (ml)
1st	0.5ml
7th	1.0ml
14th	1.5ml
21st	2.0ml

Rabbits were rested for ten days after which they were boosted with 0.5ml of the relevant antigen. Rabbits were euthanized after completing 30 days from first injection blood was collected and sera were separated.

2.6 Blood collection

Bleeding of rabbits by 14 days after the last injection. Leave the blood to clot at room temperature for 1hr. The blood left at the refrigerator overnight. Centrifugation for collection of serum which contain the antibodies. If will not be used immediately stored at -20°C. To prevent contamination, add sodium azide 0.02 %.

2.7 Sample processing

Samples of 25 grams of meat were processed in blender with 100ml of 0.85% normal saline filtered and the homogenate were centrifuged at 10000 rpm for 15 min. Then supernatant was used for analysis.

2.8 Meat analysis

Two techniques including CCIE and AGID were used to get faster, accurate and cheaper analysis of meat species

2.9 Agar Gel Immunodiffusion Test (AGID)

The Agar-gel immunodiffusion test is outstanding for its qualitative capability for exhibit differences and similarities in related proteins based upon those formation from claiming particular immunoprecipitin lines showed results because of those dispersion from claiming particular antigens and antibodies starting with wells that cut into a agar grid following they need arrived at their ideal proportions. This technique is ideally suiting to meat species protein detection. The bottom of a 6 cm Petri dish was covered with 5 ml of 1% agarose solution cooked in 50mM borate buffer, pH 8.2. After agarose gel solidification at room temperature, a cutter used to drill 7 wells of 5.5mm (1 central and 6 peripheral) in agarose and the distance between central well and peripheral well is 2.4mm. Rabbit immune-sera were added to the central well and the diluted meat extracts to the peripheral ones. Antigens for testing different raw products (50 µl) were added to the six peripheral wells. The precipitation reaction was considered positive if a pronounced positive reaction zone d in the central line between the central and the peripheral wells (between antiserum and antigen, respectively). Cover the plates and

incubate in humid chamber at room temperature and examine daily, against light and dark background, for precipitation lines at least for 48 hours.

2.10 Countercurrent immunoelectrophoresis

1.5% Agarose gel was prepared in Tris-succinic acid, pH 7.2. the gel was poured into plate and wells with a diameter of 5 mm were formed in 15 ml of agarose gel in 3 pairs on the both sides (right and left) 0.5cm apart. 20 µl of antiserum was applied into wells on anode side and 20 µl of the antigen was applied into the wells on the cathode side. Protein migration proceeded under 4 W on one plate, i.e. 15 mA at 250 V for 45 min, in the medium of Tris succinic acid. The power supply and the electrophoretic units were TEP-2 (Sevac, Czech Republic) and Bio-RAD Power Pac 3000, respectively. Results were gained after 45 mins after washing the plates in PBS, covering with Whatman paper No. 4, and staining of the plates with ethidium bromide following destaining at the destaining solution and parching at the laboratory temperature till next day.

3. RESULTS

Results of CCIE for meat samples are summarized in the table 2. The best result were obtained, where specificity and sensitivity were checked. Reactivity of meat extracts with specific antisera had given clear line of precipitation. In terms of sensitivity and specificity, good results were taken from specific antisera. The specificity of antiserum was checked while doing cross reactivity with different antigens. The reactions of antisera of donkey yielded species specific positive results with extract of donkey meat and horse meat extract yielded positive reaction with species specific antisera (horse antigen). Horse antisera yielded false positive reaction (faint line of precipitation) with donkey meat extract and vice versa.

Table 2: Cross reactivity of antisera of horse and donkey meat

Antigen (Ag)	Antisera (Ab)	Results
Donkey	Horse	Faint line of precipitation
Donkey	Donkey	Clear line of precipitation
Horse	Donkey	Faint line of precipitation
Horse	Horse	line of precipitation

Results of AGID test for homologous reactions and heterologous reactions are summarized in table 3 and 4. In the homologous reactions, the results were positive and expressed the specificity of antisera.

Table 3: Homologous reactions

Meat protein (Ag)	Antisera (Ab)	Results
Donkey	Donkey	Precipitation line shows
Horse	Horse	Precipitation line shows

In terms of heterologous reactions, the results are false positive. These reactions expressed faint line of precipitation.

Table 4: Heterologous reactions

Meat protein (Ag)	Antisera (Ab)	Results
Horse	Donkey	Faint line of precipitation
Donkey	Horse	Faint line of precipitation

4. DISCUSSION

High demand and commercial value of meat have attracted the attention of sellers to adulterate the meat for centuries. Issues like fraud detection, acquiesce with religious codes and protection of consumer drive the attention to develop the new strategies and techniques for the detection of adulterants in meat. Most common type of adulteration is the mixing of costly meat with the cheaper one; this is the matter of concern for most of the researchers and incited the research scientists to develop a sensitive and proper technique for the origin of meat that belongs to different species in different food products. This identification of meat at specie level is very significant for the consumers for various reasons; Meat frauds leads to the major economic loses and the medical point of view that some individuals might have developed allergies from specific type of meat and major one is the religious reasons. In meat during addition in formulation of product, many items are added in the form of additives that could be serious for the people consuming it from health point of view.

This study was concerned with the use of AGID (Agar Gel Immunodiffusion) technique for the detection of adulteration between the meats of two species concerning the quality of the immunogen preparation which is free of toxins. Particularly endotoxins contamination, chemical residues and other toxin contaminations that must be minimized. In my study using AGID test, no discrimination was observed between the two species meat and due to cross reactivity the false positive results was observed.

Assumed results of adulteration in meat y utilizing the techniques of AGID may ascribe to inference by the certain ingredients resent in meat products like spices, salt and soy proteins can further limits the utilization of AGID technique as state in some study [22]. Sometimes false positive results may arise due to nonspecific interactions that lead to the diffused precipitation which may be recognized by its abnormal appearance and its water solubility which is concluded [23].

If the positive reaction zone occurred in central line between the peripheral and central wells, the precipitation reaction was considered positive. This was obtained by equilibration of antigen and antiserum concentrations in order to reach the concentration between antigen and antiserum of 1:99 and 1:9 respectively. If this concentration was achieved, the zone of precipitation did not fuse and obtained as sufficiently sharp which is also detected [11].

ELISA (Enzyme Linked Immunosorbent Assay) and protein profiles have also been used for the meat identification, but the current and accurate method is countercurrent immuno-electrophoresis (CCIE). CCIE is also most accurate test as compared to AGID [24]. In this study the specificity of CCIE with three low saturated and one unsaturated antiserum was high enough to make a distinction among phylogenetically different species. This study concludes that CCIE is simple, inexpensive and very sensitive technique for the recognition of specie specific proteins in heat processed meat products.

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