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RAPID MULTIPLEX PCR-BASED IDENTIFICATION OF MEAT SPECIES (*SUS SCROFA*, *EQUUS ASINUS*, *BOS TAURUS*) IN COMMERCIAL FOOD SAMPLES

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ABSTRACT

For economic, religious, and health-related reasons, it is crucial to safeguard customers against unlawful or undesirable substitutions by evaluating the content and authenticity of food. Red meat is being replaced by the meats of cows, pigs, and donkeys. Because of their religious beliefs, Muslims and Jews abstain from eating pork and donkey meats, even in little amounts. In light of this, multiplex PCR rather than uniplex PCR is required for the quick, affordable and highly sensitive identification of the DNA origins of cow, donkey, and hog species in actual food samples. For this purpose, a total of 85 samples including blood samples, tissue samples and laboratory prepared food samples and commercial foodstuffs including mutton, beef biryani, chicken samosa, chicken pakora, haleem, nihari mutton biryani, etc. were collected from Bahawalpur and Rahim Yar Khan. DNA was extracted from all the targeted samples. In a single tube, we created the first multiplex PCR test to determine the species of cow, donkey and pork used in food items. In addition to universal 18S rRNA primers that amplify a 99 bp fragment, the assay uses specific primers [pork (*Sus scrofa*), donkey (*Equus asinus*), and cow (*Bos taurus*)] that amplify fragments of the mitochondrial COX1 (pork; 460 bp), 12S rRNA (donkey; 184 bp), and ATPase subunit 6 and 8 genes (cow; 271 bp), respectively. For all species, the assay's detection limit was 0.05%. In conclusion, this PCR technique offers an easy, quick, sensitive, accurate, and cost-effective way to identify cow, pork and donkey species in commercial food samples.

1. INTRODUCTION

Biology has proven beyond a shadow of a doubt that food was necessary for the first living thing to breathe billions of years ago. Without food, evolution, development and growth would not have been conceivable. All organisms on the planet, including both plants and animals, require nourishment in order to endure, develop and procreate. Food is the catalyst for all living things, including humans, animals and plants.

Therefore, when you eat, your body receives nutrition for the creation of energy, which keeps the body functioning. Feed is the food that domestic animals get as part of their upbringing. Muslim and Jewish populations avoid the consumption of pork and donkey meat, even in minute quantities, due to their religious faiths (Ali *et al.,* 2012). In recent decades, scientists have created a variety of methods for detecting and identifying species origin, particularly in industrial meat products, which are crucial for economic, religious and sanitary reasons.

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Food ingredients must be found and verified in order to prove food fraud definitively. From a biochemical perspective, replacement materials are frequently comparable to the primary components, making it very challenging to distinguish between the two. To prevent customers from being exposed to unidentified factors and to identify adulteration or fraudulent replacement, it is crucial to identify the species of meat products (Meyer *et al.,* 1995).

Due to dietary changes and growing knowledge of the health advantages of meat, there is a growing global desire for premium animal protein, which includes meat and products produced from it. Since the sale of food has been a commercial activity, there have always been issues surrounding food authenticity, particularly those pertaining to fraudulent adulteration and misrepresentation. Due to the critical importance of consumer health, there is a greater focus on closely monitoring food intake and verifying its authenticity in order to prevent unwanted and illegal substitutes that are motivated by economic, religious or health-related factors (Plowman and Close, 1988).

Pakistan currently lacks suitable circumstances for meat exports because there is no enforcement legislation and no readily available tests for precisely identifying the species of meat. Prohibited meat species or their hybridization with halal meat, as well as low-nutritional halal food species for financial benefit, have long been a sensitive topic in the press and public. All things considered, the nation's anti-adulteration laws present a dismal image. It is imperative that we resist this difficult situation. This means that in order to differentiate between haram species like some pig and donkey species and halal meat species like cattle scientific procedures are required. Similarly, mutton and beef are not interchangeable. However, these creatures have long been consumed in various nations, including Vietnam, Switzerland, Tahiti, a Mexico, South Korea, Taiwan and some regions of the United States (Ali *et al.,* 2014).

Regular meat fraud has spread throughout the world, perhaps endangering food safety, violating market regulations and potentially endangering public health (Li *et al.,* 2020). In recent years, methods for determining the species of meat have undergone continual evolution (Chung and Hellberg, 2020). Polymerase chain reaction, or PCR and DNA-based techniques 2 together give more accurate ways for differentiating meat species since DNA molecules are found in every cell and have a high degree of stability (Mansouri *et al.,* 2020). Real-time PCR and conventional multiplex techniques are both regarded as trustworthy procedures with excellent sensitivity and specificity for identifying meat species (Liu *et al.,* 2021).

One of the major sources of nutritional protein and an essential component of a human diet is meat. Game meats are popular because they are low in fat and cholesterol and are a wonderful source of high quality protein. What makes game meats so desirable to consume is their unique sensory appeal. Meat from animals that have not been conventionally tamed is referred to as "game meat." Wild animals that have been hunted are the source of game meat. Nonetheless, some wild species are reared in captivity, while others are either lawfully or illegally killed in the wild (Hoffman and Cawthorn, 2014).

By amplifying numerous DNA sequences, multiplex PCR, a variation on conventional PCR, can identify many species in a single tube one PCR reaction at a time (Denyingyhot *et al.,* 2022). Certain animal species, such ruminant (beef & sheep) and poultry (chicken & duck), are the most widely consumed meat sources worldwide the world, which can provide vital nutrients, particularly for the most abundant source of protein (Hossain *et al.,* 2017). However, the increased demand for protein from animals has made meat frauds in goods containing animal protein more common, which has resulted in serious worldwide problems. Whether done intentionally or accidentally, adulterating meat is against religious and ethical prohibitions as well as commercial regulations. For instance, eating pig is completely forbidden in Islam and Judaism, while Hindus are forbidden from eating beef (Li *et al.,* 2019).

2. MATERIALS AND METHODS

Samples Collection and Preparation

To optimize the simplex and multiplex PCR assays of the targeted species, the raw meats and grains of each targeted species such as cow, pork and donkey etc.

were collected from Rahim Yar Khan and 85 market food samples were collected from a different area of Bahawalpur and Rahim Yar Khan (Figure1 and Table.1). Raw and processed meat samples were cut into small pieces and immediately stored at -20°C until use. We directly transported them to the Molecular Genetics Research Laboratory (CUVAS) and stored at -20°C until the extraction of the DNA in order to prevent the enzymatic degradation of DNA.

DNA Isolation

DNA was extracted from 50-100 mg of laboratory prepared and market collected food samples using instructions and protocol of Genomic DNA Purification Tissue and Blood Kit (Thermo-Scientific) and DNeasy® Kit (Germany) as per instructions of protocol given in the kit. DNA concentration (quantification) in a solution was measured using a Gel Doc XR+ with imaging Lab instrument. It produced high-quality photographs with exceptional sensitivity and resolution.

Sensitivity and Validation of Laboratory Prepared Samples

Meats and grains from cattle, pork, donkeys, and other species were combined in varying percentages and cooked in a lab to authenticate the market food samples (Table 2). The sensitivity test was assessed by mixing and cooking compound DNAs from all species (cow, goat, buffalo, pig, donkey, etc.) in varying percentages up to 0.05% (Table 3). Ultimately, these samples' DNA was isolated and kept at -20°C until it was needed.

Oligonucleotides Primers

Specific gene loci, such as the COX1 gene for pork, the 16S rRNA for donkeys, and the ATPase subunits 6 and 8 for cows, were amplified using oligonucleotide primers. Next, we used the BLAST and NCBI (National Center for Biotechnology Information) tools to verify the specificity of the DNAs in the DNA databank (Table. 4). All the primers were synthesized by Molecular Biology Products Company, USA.

Simplex qualitative PCR

For PCR amplification, a final volume of 20 µl was utilized, which included 1 µl of each primer, 3 µl of DNA template, and WizPureTM PCR 2x Master mix. The following cycling settings were used in a Thermocycler Techne to perform amplification: After a 5-minute initial heat denaturation stage at 95 °C, 35 cycles were programmed: A 5-minute extension at 72°C, 30-second intervals at 95°C, 59–61°C, and 72°C for one minute were also employed.

Multiplex qualitative PCR

A single-step multiplex PCR was developed for the simultaneous identification of all species using each primer set that had been previously selected for the simplex PCR. To a total amount of 20 µl for PCR amplification, 0.4 µl of each primer, 1 µl of DNA template, and the WizPureTM PCR 2x Master mix were added. The amplification procedure was carried out in a Thermocycler Techne with the following cycling parameters: Following an initial heat denaturation stage at 95 °C for 5 min, 35 cycles were planned: 95 °C for 30 s, 59–61 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min.

3. RESULTS

DNA Extraction

DNA was extracted from laboratory prepared samples as well as from samples obtained from three districts. Different DNA extraction kits were used according to the manufacturers' instructions. The total extracted DNA was labeled and preserved at -20°C for subsequent PCR use.

Optimization of Simplex PCR

For the purpose of identifying adulteration of cow, pig, and donkey in food and feed, a PCR technique that uses species-specific primers to amplify DNA has been selected. To confirm the specificity and sensitivity of the primers used in the initial PCR studies, simplex PCR was optimized and performed on DNA samples isolated from cooked meats. For the cow, pig, and donkey species, respectively, the primers produced distinct fragments of 271 bp, 460 bp, and 184 bp (Figure 3). Each pair of primers was run through a simplex PCR with non-target species in order to look for potential cross-reactions; no false positive amplification in related species was seen.

Simplex and Multiplex PCR Specificity and Optimization

First, simplex PCR was optimized, followed by the optimization of multiplex PCR using the same primers

and DNA extracted from cooked meats of cow, pork, donkey, etc., after several repetitions (Figure 4).

Sensitivity test of Multiplex PCR

The sensitivity of the multiplex PCR, targeting the same species' DNAs, was evaluated down to a minimum concentration of 0.05% (Figure 5).

Validation of the Assay on labortory Prepared Samples

To validate the market food samples, including meats such as cow, pork and donkey, as well as various grains, mixtures of these species were prepared in different percentages and cooked in the laboratory. DNA was then extracted from these samples, and a developed multiplex PCR was performed for validation (Figure 6).

Application of Multiplex PCR Assay

We have applied our developed assay on 85 commercial food samples. We had not found any contamination with pork and donkey in 85 collected samples, while we had found 10% adulteration that may be intentionally or un-intentionally by producers.

4. DISCUSSION

In order to assess cocktail food products and verify the authenticity of labels, this study emphasizes the urgent necessity for rapid, sensitive, accurate, and reasonably priced identification techniques, particularly when it comes to distinguishing donkey, pork and cattle species. The inclusion of donkey and hog meat (which has religious overtones), the health concerns (for both children and adults), and the adulteration of expensive meats (like beef) with less expensive ones (like chicken) all underscore the need for advanced species identification procedures in sausages. Previous research have reported species-specific PCR-based techniques to detect animal species in food items (Palle *et al.*, 2014). However, compared to earlier techniques, this PCR test offers a number of benefits. The triplex PCR assay can quickly and easily identify donkey, pig and beef in a variety of food products without the need for additional reagents, specific knowledge, or peculiar equipment. Because it can identify species-specific DNA, simple agarose gel analysis is a cost-effective first screening technique for large-scale applications.

The detection limit of our assay is 0.005%, demonstrating its high sensitivity and reliability. In comparison, other researchers have reported higher detection limits for meat species identification in food products, with a minimum detection limit of 0.1% 0.5% (Ha *et al.,* 2006). This study used the polymerase chain reaction, also called PCR, to detect several adulterants (donkey or pig) in an experiment mixture of fresh minced beef with well-known formulas. Multiplex PCR has steadily gained popularity over simplex PCR due to numerous benefits, which include faster reaction times, reduced costs, and improved detection efficiency in just one reaction hole. Safdar (2015) demonstrated a quadruplex PCR method with a detection limit of 0.01% DNA, capable of simultaneously identifying bovine, ovine, caprine, and fish species in feedstuffs (Safdar and Junejo, 2015).

According to (Karabasanavar *et al.,* 2014) The traditional multiplex PCR method created in this work was simple to use and didn't require expensive reagents or equipment, making it affordable for nearly every laboratory. Three easy steps extracting DNA, executing a PCR amplifcation program, and conducting agarose gel electrophoresis could be completed in a half-day by one person. Since mitochondrial DNA was present in numerous copies within cells, the mitochondrial 12S rRNA gene was chosen as an appropriate molecular marker. Based on our findings, every single pair of species-specific primers was extremely specific.

One of multiplex PCR's drawbacks is the possibility of primer compatibility problems, which might result in less sensitive or non-specific amplification. Furthermore, it can be difficult to optimize reaction conditions for several targets at once, and there is a chance that some targets will be amplified more often than others, which might reduce the assay's overall accuracy.

5. CONCLUSION

The developed multiplex PCR assay provides a rapid, sensitive, specific, and cost-effective approach for the simultaneous identification of *Bos taurus, Sus scrofa*, and *Equus asinus* DNA in raw, processed, and commercial food products. The assay demonstrated excellent specificity without cross-reactivity and achieved a detection limit of 0.05%, confirming its suitability for detecting low levels of meat

adulteration. Validation using laboratory-prepared mixtures and 85 commercial food samples further demonstrated its reliability for routine food authentication. This method represents a practical tool for regulatory agencies, food industries, and quality control laboratories to monitor meat authenticity, prevent food fraud, and protect consumer interests while addressing economic, public health, and religious concerns.

6. ETHICAL STATEMENT

No animals were harmed or disturbed during this research.

7. CONFLICT OF INTEREST

All authors have declared that there is no conflict of interest regarding the publication of this article.

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Table 1. Collection of samples from different cities of Bahawalpur and Rahim Yar Khan

Sr.no	District	Areas	Sample Types	Total sample
1	**Bahawalpur**	Yazman	Kofte, Somsa, beef	10
		Bwp city	Biryani (Chicken), Haleem, Alo keema	14
		Hasilpur	Chicken Samosa, beef, haleem	12
		Ahmedpur East	Pakora, biryani, mutton	16
	Total			**52**
3	**Rahim Yar Khan**	RYK city	Haleem (Beef), biryani, keema gosht	08
		Khanpur	Keema Goste, Haleem chicken	04
		Liaquatpur	Nihari (Beef), nihari chicken, somsa	09
		Firoza	Haleem (Beef), biryani, pokra	07
		Chani Goth	Alo Keema, mater keema, beef	05
	Total			**33**
	Sample (n)			**85**

Table 2. Preparation of samples for Validation

Buffalo (NC) %	Donkey %	Pork %	Cow %	Goat (NC) %
10	80	55	30	70
40	0.05	20	0	50
60	10	0	45	35
100	0	40	65	0

Note: NC (negative control)

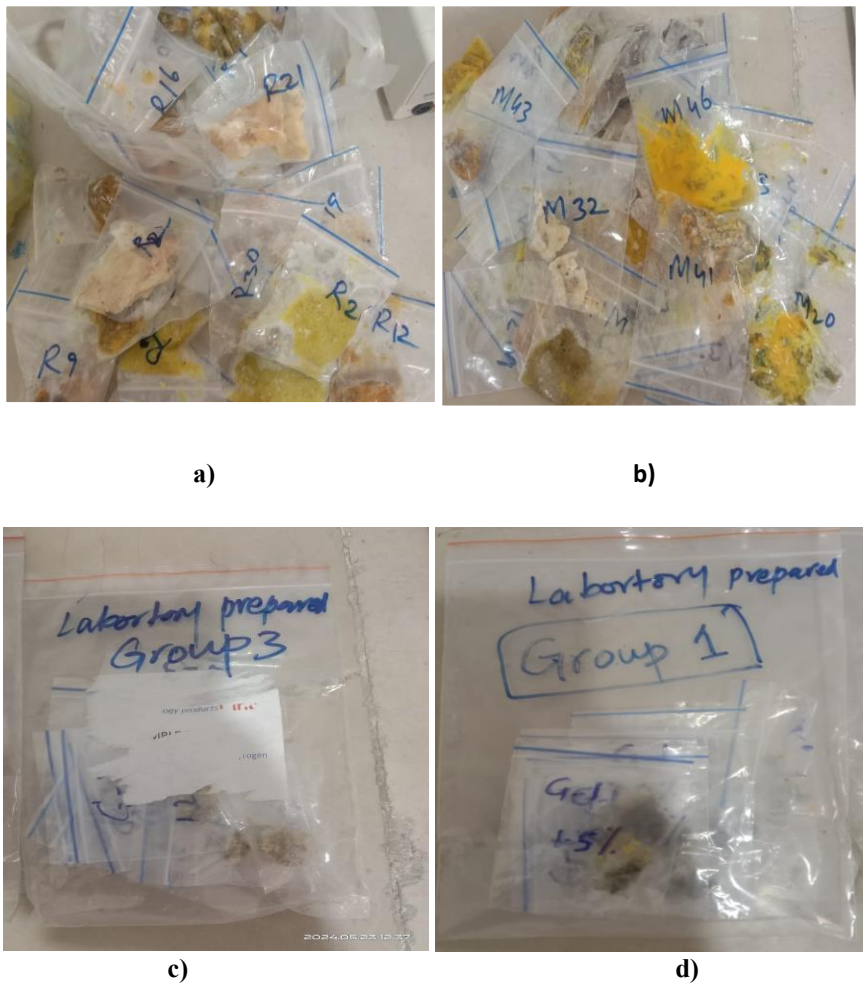
Table 3. Preparation of samples for sensitivity

Buffalo (NC) %	Donkey %	Pork %	Cow %	Goat (NC) %
22	22	22	22	12
11	11	11	11	56
5	5	5	5	80
3	3	3	3	88
2	2	2	2	92
1.5	1.5	1.5	1.5	94
1	1	1	1	96
0.75	0.75	0.75	0.75	97
0.05	0.05	0.05	0.05	99.8

Note: NC (negative control)

Table 4. Targeted species primers use in this study

Primers	Genes	Oligonucleotides primers 5' → 3'	Amplicons (bp)
Pork *(Sus scrofa)*	*COX1*	F: CTACTATCCCTGCCAGTT	460
		R: GAATAGGAAGATGAAGCC	
Donkey *(Equus assinus)*	*12S rRNA*	F: GAACAAGAACTCAACCCAAACGG	184
		R: CTTTCATATGTTTGGATCATGGTTTTG	
Cow *(Bos Taurus)*	*ATPase subunit 6&8*	F: GCCATATACTCTCCTTGGTGACA	271
		R: GTAGGCTTGGGAATAGTACGA	
Eukaryotes	*18S rRNA*	F: AGGATCCATTGGAGGGCAAGT	99
		R: TCCAACTACGAGCTTTTTAACTGCA	



a) **b)**

c) **d)**

Figure 1. Collected samples from market and Laboratory prepared samples

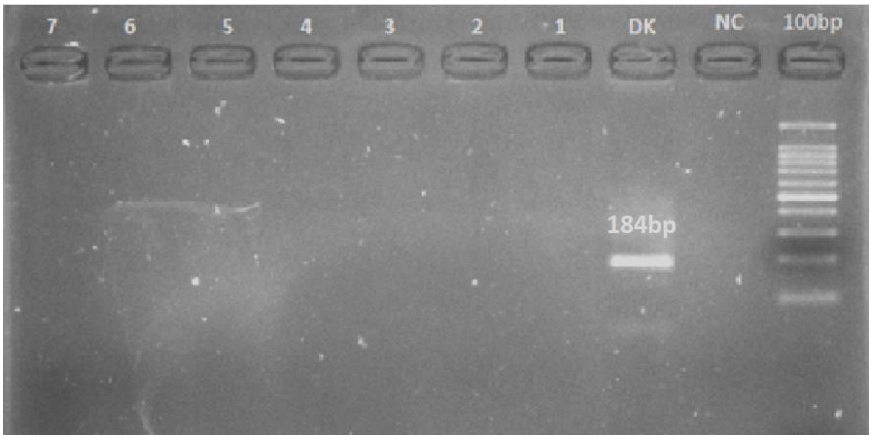


Figure 2. A 100bp: Ladder, NC: Negative Control (Everything without primer of DK), DK: Donkey (Positive Control), 1. Cow 2. buffalo 3. Sheep 4. Goat 5. Pork 6. Cat 7. Dog

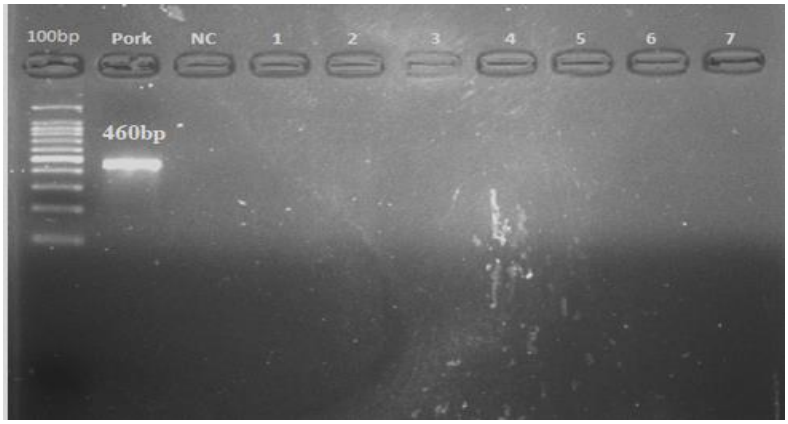


Figure 2. B 100bp: Ladder, NC: Negative Control (Everything without primer of Pork), Pork (Positive Control), 1. Cow 2. buffalo 3. Sheep 4. Goat 5. Donkey 6. Cat 7. Dog

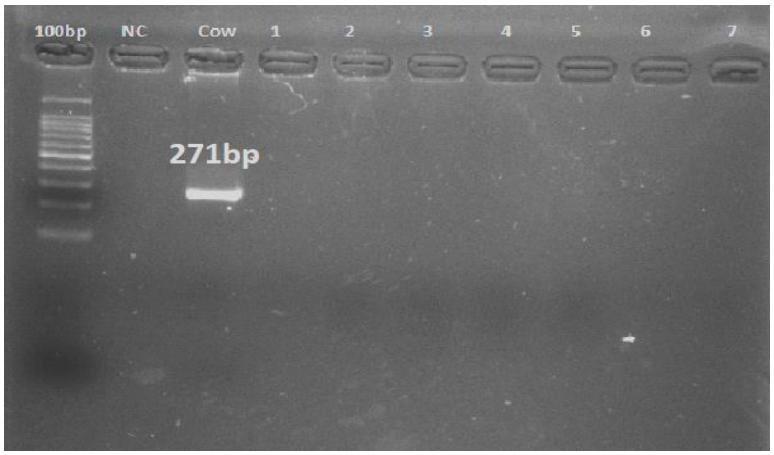


Figure 2. C 100bp: Ladder, NC: Negative Control (Everything without primer of cow), Cow (Positive Control), 1. pork 2. buffalo 3. Sheep 4. Goat 5. Donkey 6. Cat 7. Dog

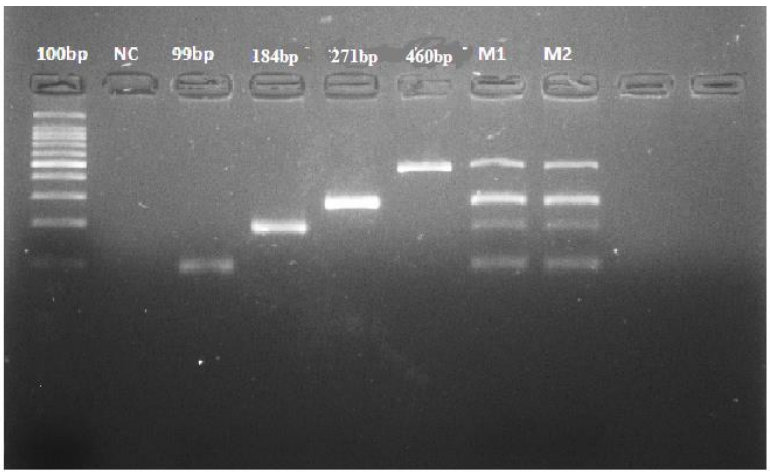


Figure 3. 100bp: Ladder, NC: Negative Control (Everything without primer of cow), Cow (Positive Control), Universal primer (99bp), Donkey (184bp), Cow (271bp), Pork (460bp). M1; Multiplex PCR 1, M2; Multiplex PCR 2 (Repeat).

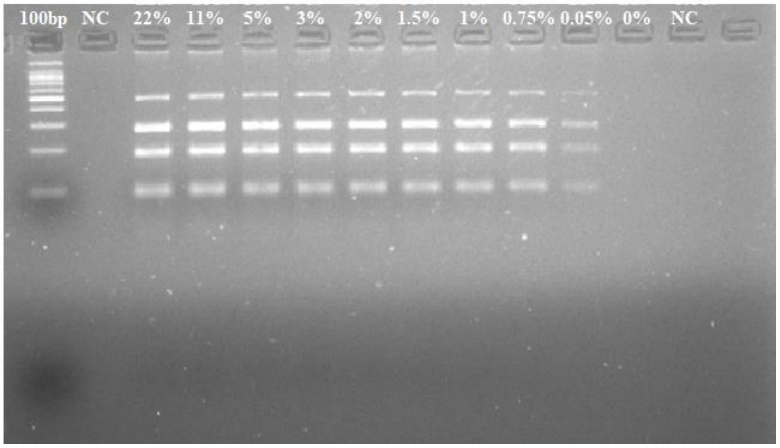


Figure 4. Evaluation of multiplex PCR assay sensitivity for universal, cow, donkey and pork in cooked labortaty prepared samples; M: Marker 100-bp. NC: Negative control (reagents with primers without DNAs); Eu: Eukaryotic DNA positive control. (1) 22%, (2) 11%, (3) 5%, (4) 3%, (5) 2%, (6) 1.5%, (7) 1% (8) 0.75% (9) 0.05% (10) 0%

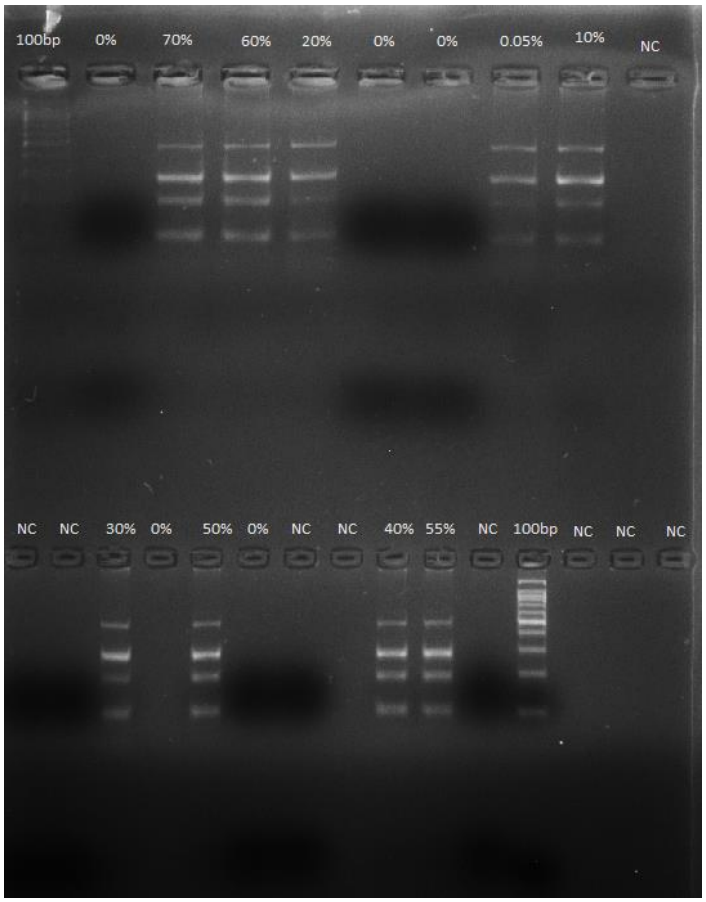


Figure 6. Evaluation of multiplex PCR assay validity was performed on cow, donkey and pork in cooked labortaty prepared samples; M: Marker 100-bp. NC: Negative control (reagents with primers without DNAs).