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Robust detection of cow and female buffalo DNA through Real-Time PCR assay

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ABSTRACT

Cow, *Bos taurus*, and female buffalo, *Bubalus bubalis,* are considered sacred animals that are a part of rural livelihood in India. The purity of products from these bovine species has significant sentimental implications in the dairy and meat industry. Therefore, the mitochondrial DNA and the sex origin, targeting the X and Y chromosomes from these bovine species, were selected to design three multiplex real-time probe PCR assays: Hi-PCR® Cow Detection Kit (MBPCR184), Hi-PCR® Buffalo Detection Kit (MBPCR185) and Hi-PCR® Cattle Sex Determination Kit (MBPCR186). Scientific Working Group on DNA Analysis Methods (SWGDAM) guidelines were followed to perform different studies using reference control DNAs. An Internal Reagent Control (IRC) was part of every assay, thus ensuring a successful reaction. The assays were 100% specific, with no cross-amplification of the two bovine species. The amplification of the X chromosomal target was observed for male and female DNAs, whereas Y chromosome amplification was observed only for the male DNA. The assays were 100% specific to the target genes in these organisms with no non-specificity towards any other targets or organisms. The limit of detection for sex determination was 0.01 ng/μl, whereas the differential capability of the assay was 3 copies/μl and 30 copies/μl for *Bos taurus* and *Bubalus bubalis*, respectively. The assays were reproducible at 1 ng/μl genomic DNA with 95% CI. The assays are open and compatible with other brands of Real-Time PCR systems used in forensic labs. The experiments presented here verify that the developed real-time PCR assays are robust, produce reliable and reproducible results for detection and differentiation of *Bos taurus* and *Bubalus bubalis* and their sex even at low DNA concentrations.

1. Introduction

Adulteration of food products with cheaper alternatives that are available *en masse* is observed worldwide, leading to regulatory noncompliance, religious concerns, or allergic issues [1]. Following the European scandal of horse meat in 2013 [2], adulteration and species substitution irregularities have received full attention worldwide. Southeast Asian countries where certain animals are worshipped as avatars of God present a unique situation with adulterated meat. In India, owing to the cattle's status and religious sentiments attached with it cow slaughter is legally prohibited with legislation in place throughout most states and territories of India [3]. In such markets, the identity of the source of bovine meat is very critical for sociopolitical as well as scientific reasons.

Analytical tools are necessary to identify the fraudulent use of different species as food substitution. In cases, where the food changes drastically after processing, thus hampering the visual identification of the product, molecular analysis becomes essential [1]. Quantitative real-time PCR (qPCR) is a method of choice for simultaneous identification of species and quantification of nucleic acids with robust sensitivity, specificity, and accuracy [4].

In the present study, developmental validation studies for three new multiplex real-time probe PCR assay kits (Hi-PCR® Cow Detection Kit, MBPCR184; Hi-PCR® Buffalo Detection Kit, MBPCR185; and Hi-PCR® Cattle Sex Determination Kit, MBPCR186) to detect the mitochondrial DNA (mtDNA) and the sex origin targeting the X and Y chromosomes from *Bos taurus* and *Bubalus bubalis* were carried out. In addition, various parametric studies viz., PCR conditions, sensitivity, species specificity, precision, and accuracy, intermachine assessment, contamination assessment, and sample studies were performed following Validation Guidelines for DNA Analysis Methods [5].

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2. Material studied, methods, techniques

2.1. Primer and probe design

The primers and probes specific to the cytochrome *b* of mtDNA and the X and Y chromosome from *Bos taurus* and *Bubalus bubalis* were designed with PrimerQuest™ Tool [6]. In addition, an Internal Reagent Control (IRC) was multiplexed with the three assays.

2.2. DNA references

Certified DNA references of the cow, *Bos taurus*, buffalo, *Bubalus bubalis*, Bovine Genomic DNA (male and female), and other animals (Zyagen, Inc.), human (male and female) (Promega Corporation), and microbial DNA (American Type Culture Collection) were used.

2.3. Real-time PCR

Real-time PCR reactions were performed using the Insta Q96® 6.0 Real-Time PCR System (LA1074, HiMedia Laboratories) with an optimized concentration of each specific primer and probe. The master mix was prepared by combining 12.5 µl of Hi-Quanti 2X Realtime PCR Master Mix (MBT181, HiMedia Laboratories), the optimized concentration of each specific primer, probe, and template DNA. The cycling conditions were optimized to complete the reaction in < 60 min.

2.4. SWGDAM-recommended studies

Specificity, sensitivity, precision and accuracy, contamination assessment, intermachine assessment, and sample studies were conducted following SWGDAM guidelines [5]. For sample studies, dairy and meat samples were collected from local market, and DNA was extracted using HiPurA® Multi sample Pre-Filled Plates for Insta NX® Mag32 MB554MPF32200 on Insta NX® Mag32 (LA1096, HiMedia Laboratories).

3. Results

3.1. Species specificity

The developed assay was highly specific for bovine DNA with amplification observed only for cow and buffalo DNA samples. No amplification was detected for other animals, humans, and microbial DNA (Table 1).

Table 1
Details of DNA templates used for specificity studies.

Sr. No	Name	Make	Cow	Buffalo	Cattle sex
1	Cow (*Bos taurus*) Blood Genomic DNA	Zyagen	+	–	XX
2	Buffalo (*Bubalus bubalis*) Blood Genomic DNA		–	+	XX
3	Bovine Genomic DNA (Male)		+	–	XY
4	Bovine Genomic DNA (Female)		+	–	XX
5	Other animals DNA*		–	–	–
16	Human Female DNA	Promega	–	–	–
17	Human Male DNA		–	–	–
18	*Escherichia coli* 25922	ATCC	–	–	–
19	*Klebsiella pneumoniae* 13883		–	–	–
20	*Pseudomonas aeruginosa* 27853		–	–	–
21	*Staphylococcus aureus* 25923		–	–	–
22	*Streptococcus pneumoniae* 49619		–	–	–
23	*Enterococcus faecalis* 29212		–	–	–
24	Saccharomyces cerevisiae 9763		–	–	–
25	*Candida albicans* 10231		–	–	–

Legend: * - Dog, Cat, Horse, Rat, Rabbit, Pig, Chicken, Sheep, Mouse, and Goat ATCC – American Type Culture Collection, (+) - Positive, (-) – Negative, XY – Male, XX – Female

3.2. Male specificity

The detection assay clearly identified male and female DNA. In the sex determination assay, X chromosome amplification was detected for both the bovine male and female DNA, whereas Y chromosome amplification was observed only for the bovine male DNA (Table 1).

3.3. Sensitivity

The analytical sensitivity of the assay or the limit of detection (LOD) of the assays was determined to be as low as 3 copies/µl tested with the respective reference DNA. The genomic DNA was serially diluted up to 0.01 ng/µl from 1 ng/µl (Table 2).

3.4. Precision and accuracy

The assay was 100% reproducible. The reproducibility and reliability of a PCR test require that the test runs with the same results each time. Therefore, the reproducibility of the assays was assessed by amplifying a series of 22 replicates at a given DNA concentration (Table 2).

3.5. Contamination assessment

The assays were highly accurate and showed very little background noise. No detectable signal was observed in the No Template Control (NTC) reactions for either cow, buffalo, or X or Y chromosome targets for 97.87% (46/47) of the reactions. The single positive control reaction showed the desired amplification at 1 ng/µl of the template DNA concentration.

3.6. Intermachine assessment

The developed assays were highly reproducible on other Real-Time PCR system. The optimized PCR conditions of Insta Q96® 6.0 Real-Time PCR System (LA1074, HiMedia Laboratories) were replicated in QuantStudio™ 5 Real-Time PCR System (Thermo Fisher) with high performance and efficiency (Fig. 1).

4. Discussion

Real-Time PCR-based methods play an essential role in identifying adulteration and can be used to authenticate the food products [7,8]. The present study developed three multiplex real-time PCR assays to detect cow and buffalo mtDNA and X and Y chromosome nuclear DNA. Mitochondrial DNA (mtDNA) is a circular molecule with a covalent ring structure resulting in superior resistance to fragmentation and degradation. Further, the cytochrome *b* gene sequences are widely used as a molecular chronometer for the identification of eukaryotic species. It is, therefore, widely used for species identification [9].

The cytochrome *b* sequences and the X and Y chromosome sequences from *Bos taurus* and *Bubalus bubalis* were obtained from the GenBank database and aligned with MEGA X [10]. Primers and probes were designed with PrimerQuest™ Tool [6]. The probes for detection of *Bos*

Table 2
Analytical Sensitivity and Reproducibility of the three real-time PCR assays.

Assay Name	Limit of Detection		No. of replicates (1 ng/µl)	
			Tested	Amplified
Cytochrome b of Cow	0.01 ng/µl	3 copies/µl	22	22
Cytochrome b of Buffalo	0.1 ng/µl	30 copies/µl	22	22
X chromosome	0.01 ng/µl	3 copies/µl	22	22
Y chromosome	0.01 ng/µl	3 copies/µl	22	22

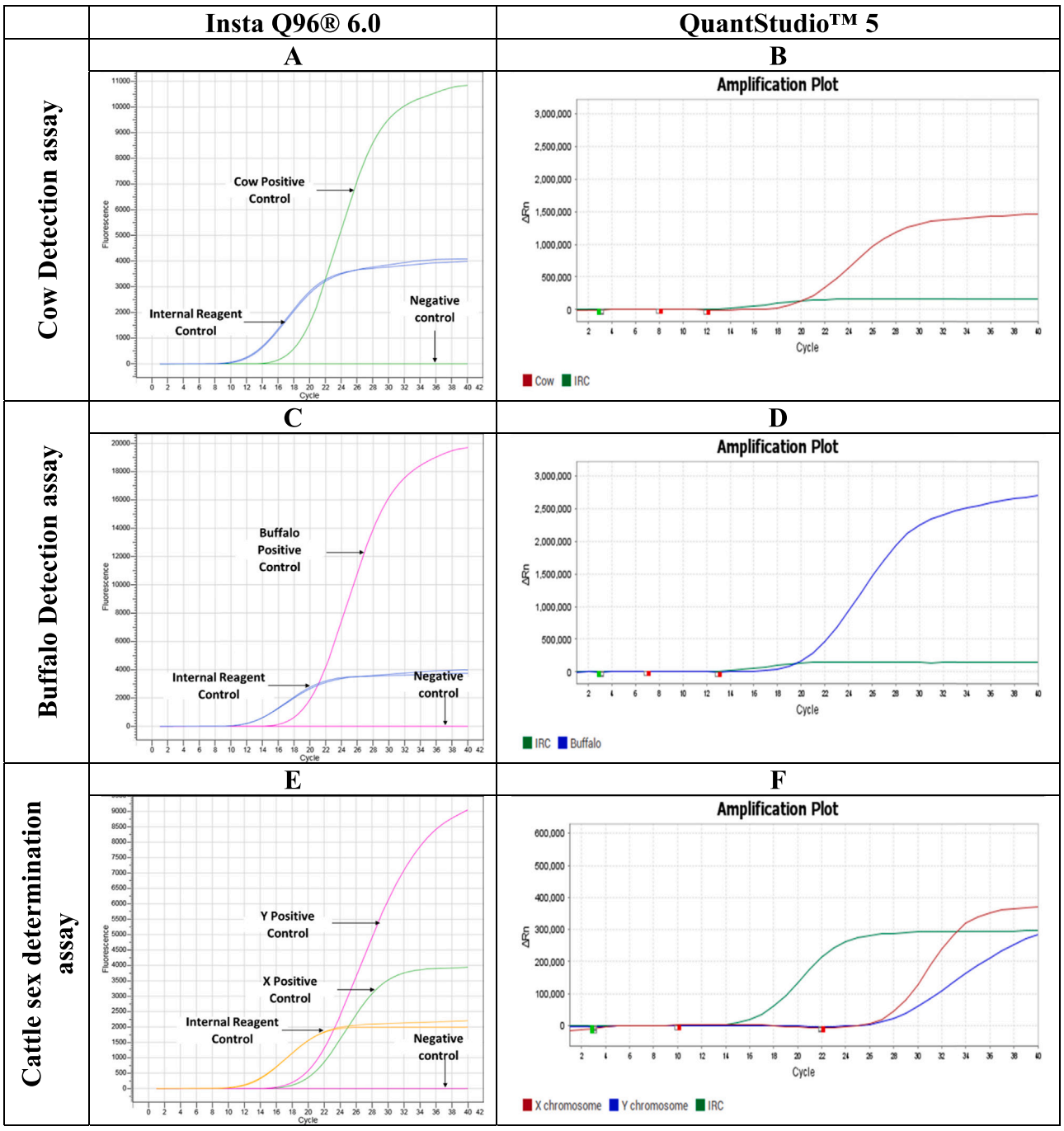

Fig. 1. Representative amplification curves for the real-time PCR assay.

taurus and *Bubalus bubalis* were tagged with FAM dye each. The probes detecting X and Y chromosomes were tagged with FAM and JOE dyes, respectively. The three assays were multiplexed with an IRC tagged with JOE dye to detect *Bos taurus* and *Bubalus bubalis* while tagged with TexasRed dye for determination of sex origin. The IRC is an integral part of a PCR assay and can be used to determine if an assay result is reliable. The primers and probes used for the IRC were non-reactive to either target genes and consistently generated a threshold cycle (Ct) in the samples [11].

Tests of certified DNA references showed that the three assays were highly specific and discriminatory. The results from the cow and buffalo primers-probes showed no cross-amplification with either of the bovine DNA. Exclusivity detection of the male samples and differentiation of the female samples were observed. Overall, the assays were 100% specific to identify their specific targets.

The cow and sex determination assays could detect their respective target DNA at quantities as low as 3 copies/μl (0.01 ng/μl). However, the detection limit of the buffalo assay was 30 copies/μl (0.1 ng/μl). These low-level detectable limits are highly appropriate for authenticity purposes [12].

PCR detection assays were performed to monitor the presence of contamination in laboratory environments or assay reagents. Statistical confidence intervals (95%) were achieved, with no amplification observed for most replicates. Surprisingly, one replicate showed false positive amplification, which could be due to the enzymatic decomposition of PCR products or the generation of spurious signals [13]. However, such low-level spurious amplification did not impact the accuracy of the developed assay.

With different real-time PCR systems available, it is vital to ensure the kits are compliant with systems being widely used in forensic labs across India. Therefore, all three assays were tested and were found compatible with QuantStudio™ 5 Real-Time PCR System (Thermo Fisher).

5. Conclusion

The present study demonstrates the efficiency, specificity, reproducibility, and robustness of three newly developed multiplex PCR systems targeting the mtDNA and the sex origin of bovine livestock. A one-stop solution is available from DNA extraction using HiPurA® Multi sample Pre-Filled Plates for Insta NX® Mag32 MB554MPF32200 on Insta NX® Mag32 (LA1096, HiMedia Laboratories to multiplex probe PCR assays on Insta Q96® 6.0 Real-Time PCR System (LA1074, HiMedia Laboratories) and compatible with QuantStudio™ 5 Real-Time PCR System (Thermo Fisher). We conclude that these systems offer promising applications for amplifying the targeted mtDNA and the sex chromosomes and can be applied to routine forensic investigations of meat adulteration.

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