



Meat species identification using DNA based molecular techniques

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ABSTRACT

Techniques based on nucleotide sequencing and restriction fragment length polymorphism (RFLP) targeting mitochondrial (mt) cytochrome B (cyt B) gene were developed for identification of species of meat in the present study. Universal primers flanking mt cyt B gene were designed to yield a single amplicon of 450 bp size upon polymerase chain reaction (PCR) in all meat animal species. Sequencing of mt cyt B gene of cattle (*Bos indicus*/*Bos taurus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hircus*), chicken (*Gallus gallus*) and pig (*Sus scrofa*) was carried out and resultant sequences were aligned using Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnological Information (NCBI) to establish the homogeneity and divergence in nucleotides among these species to ascertain unambiguous identification of origin of a meat species. Closely related species like cattle & buffalo; and sheep & goat could be differentiated conclusively by sequence analysis. Although nucleotide sequencing is a highly accurate technique, it is time consuming and costly. Hence, an economical, rapid and reliable RFLP method was developed. Nucleotide sequences of amplified fragments were mapped for restriction enzyme and *MspI* enzyme was found to possess restriction site only in cattle (198 and 252 bp) and pig (389 and 61 bp) but not in other species studied that enabled development of an RFLP technique for the identification of beef and pork simultaneously. The PCR-RFLP technique was found applicable even in the cooked and admixed meat samples.

Key words: Cytochrome B, PCR, RFLP, Sequencing, Speciation

Fraudulent substitutions of cherished and valued meats by easily available low priced ones have been reported worldwide. The scandal of adulteration of horse meat in beef in the year 2013 in Europe is one such example of widespread practice of fraudulence in meat trade. In India, slaughter of cattle (*Bos indicus*/*Bos taurus*) is restricted on religious ground. Further, there is a ban on the export of beef (from cattle) but export of buffalo meat is permitted. Misrepresentations violating such beliefs and guidelines are punishable under law. Owing to the potential health risk linked to the consumption of beef associated with transmissible spongiform encephalopathy (Ahmed *et al.* 2007) especially in Europe has led to ban on the use of cattle (ruminant) derived products as animal feed. It is essential to ensure meat authenticity for protecting consumers sentiments, implementation of legislations on

labeling and to protect fair trade (Singh and Sachan 2011).

DNA based molecular techniques have been increasingly relied in the recent times owing to higher stability of DNA that can even sustain thermal processing (Karabasanavar *et al.* 2011; Wang *et al.* 2013). Mitochondrial targets are preferred over nuclear genes due to high copy number and conserved nature of mitochondrial sequences in all the cells there by aiding in the enhanced sensitivity and preciseness of PCR based assays not only in fresh meat but even in the processed meat products (Fajardo *et al.* 2010). Of the several mitochondrial targets, most widely used targets for species identification include cytochrome B gene (Murugaiah *et al.* 2009). Present study describes application of sequence analysis and PCR-RFLP of mitochondrial cytochrome B gene for the authentication of meat (fresh and cooked) and detection of adulteration buffalo meat with beef (cattle).

MATERIALS AND METHODS

Meat samples: Meat samples of cattle (*Bos indicus*), buffalo (*Bubalus bubalis*), goat (*Capra hircus*), and sheep (*Ovis aries*) were collected from municipal slaughter house, Chengicherla, Hyderabad. Chicken (*Gallus gallus*) and pig (*Sus scrofa*) meat samples were collected from the authentic retail shops located in Hyderabad city, Telangana, India. Similarly, blood samples of different cattle breeds (Deoni, Red Kandahari and Jersey cross-breeds) were collected from Teaching Veterinary Clinical Complex, Veterinary College,

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DNA extraction: The DNA from meat samples was extracted as per the method described by Chikuni *et al.* (1990). DNA from blood was extracted by following the procedure described by Sambrook and Russel (2001).

Thermal processing of beef samples: Fresh raw meat samples were subjected to thermal processing at 72° C in hot air oven for 30 min, steam cooking at 90°C for 30 min and autoclaving at 121° C at 15 psi pressure for 30 min.

Preparation of meat mixture: In order to verify detection of meat adulteration using PCR-RFLP of mitochondrial cytochrome B gene, various combinations of meat mixtures of beef in buffalo meat were prepared *viz.*, 5:95, 10:90, 20:80, 40:60, 50:50, 60:40, 80:20, 90:10 and 95:5 (w/w). From mixed meats, 10 mg each of meat was taken from ten different portions and a total of 100 mg meat was subjected for DNA extraction.

Designing of primers: Universal primers targeting mitochondrial cytochrome B gene were designed by analyzing nucleotide sequences downloaded from the GenBank database (www.ncbi.nlm.nih.gov) of National Centre for Biotechnology Information (NCBI). Conserved region common to all the studied species was identified and oligonucleotide primers were designed using online NCBI software. Sequence of primers designed were - Forward 5' - CCC TTA CCC GAT TCT TCG CTT TCC A - 3' and Reverse 52 - GGC ATT GGC TGA GTG GTC GGA - 3'.

Optimization of PCR: The PCR conditions were optimized to obtain a desired amplicon of 450 bp from meat samples. The cycling conditions comprised of initial denaturation (94° C, 5 min) followed by 29 cycles of denaturation (94° C, 45 s), primer annealing (58° C, 60 s) and extension (72° C, 60 s); and the final extension (72° C, 10 min). The size of the amplicons was determined by agarose gel electrophoresis as described by Sambrook and Russel (2001).

Nucleotide sequencing and analysis of cytochrome B gene: The PCR amplicons were custom sequenced and aligned using Basic Local Alignment Search Tool (BLAST) of NCBI.

Polymerase Chain Reaction- Restriction Fragment Length Polymorphism (PCR-RFLP): The PCR amplicons of mt cyt B gene of samples were subjected to restriction enzyme digestion with *MspI* restriction enzyme as per the supplier's instruction.

RESULTS AND DISCUSSION

Approaches followed for developing speciation techniques included sequence analysis which enables identification of species of all meat animals and Polymerase Chain reaction - Restriction Fragment Length Polymorphism (PCR - RFLP) for specific identification of beef and pork since both cattle and pig meat are religiously restricted/ prohibited for slaughter. Newly designed universal primers targeting partial sequence of mitochondrial cytochrome B gene amplified 450 bp

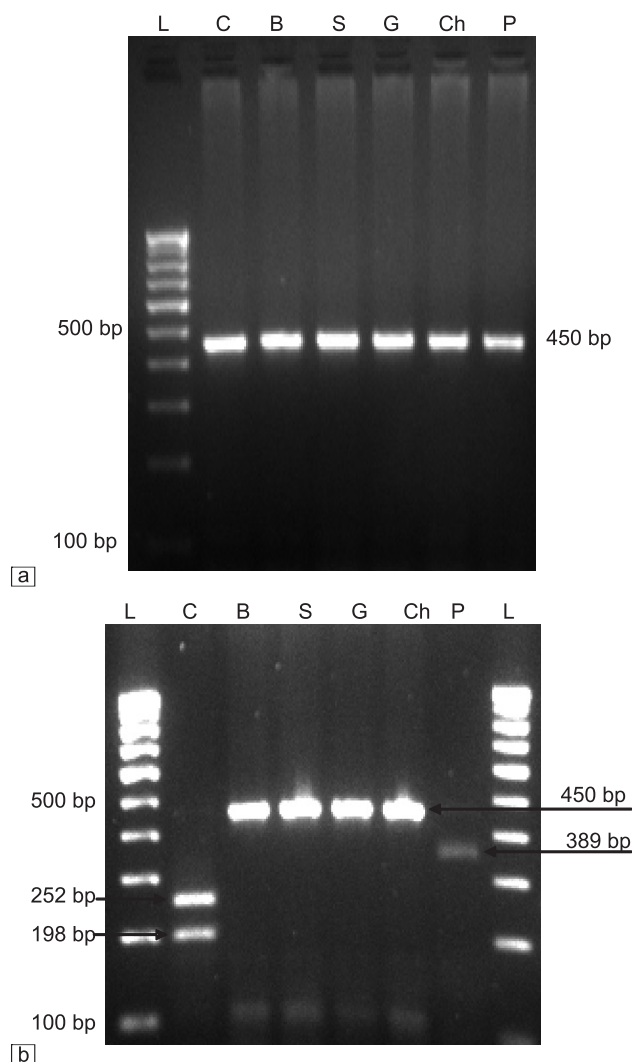


Fig. 1. (a) PCR amplification of mt cytochrome B gene of different meat species; (b) PCR-RFLP of mt cytochrome B gene in different meat species with *MspI* restriction enzyme. Lane L: 100 bp ladder; C: cattle; B: buffalo; S: sheep; G: goat; Ch: chicken; P: pig

fragment of mitochondrial cytochrome B gene in all the samples tested *viz.* cattle, buffalo, sheep, goat, chicken and pig (Fig. 1a). Nucleotide sequences of mitochondrial DNA are more conserved within a species than nuclear DNA and more number of mitochondria have been found per cell in animals which increases the chances of survival of mitochondrial DNA in different processing conditions to which meat is often subjected to (Bellagamba *et al.* 2001). Rate of mutation is high in mitochondrial DNA (ten times greater than nuclear DNA) which facilitates quick accumulation of mutations allowing the discrimination of closely related species (Tobe *et al.* 2010) than the genomic DNA and hence mt cytochrome B gene have been extensively used for species authentication (Kimwele *et al.* 2012; Wang *et al.* 2013).

Representative PCR amplicons were sequenced and nucleotide sequences of cattle (*Bos indicus*), buffalo (*Bubalus bubalis*), sheep (*Ovis aries*), goat (*Capra hircus*),

Table 1. Nucleotide sequence similarity (upper triangle, %) and divergences (lower triangle, %) of mitochondrial cytochrome B gene of different meat animal species

Percent divergence	Percent similarity							
	1	2	3	4	5	6		
1		86.8	81.4	81.4	79.6	69.8	1	<i>Bos indicus</i> (KC415731)
2	14.9		84.1	81.7	80.8	71.0	2	<i>Bubalus bubalis</i> (KC415732)
3	19.3	16.7		85.9	76.6	69.8	3	<i>Ovis aries</i> (KC415729)
4	16.6	16.8	13.8		75.7	69.8	4	<i>Capra hircus</i> (KC415728)
5	22.7	20.3	24.8	23.6		73.4	5	<i>Sus scrofa</i> (KC415733)
6	34.4	31.9	32.4	30.9	29.2		6	<i>Gallus gallus</i> (KC415730)
	1	2	3	4	5	6		

chicken (*Gallus gallus*) and pig (*Sus scrofa*) were submitted to the European Molecular Biology Laboratory (EMBL) nucleotide sequence database (Accession Numbers: KC415728 to KC415733). Retrieved sequences were analysed using BLAST (www.ncbi.nlm.nih.gov/BLAST) online tool and Clustal Omega (1.1.0) programme (embl.ebi.ac.uk) for cattle, buffalo, sheep, goat, chicken and pig (Fig. 3). Based on the alignment and divergence of the nucleotide sequences (Table 1) with the sequences of different meat animal species, given meat species was confirmed. Resultant cattle mt cyt B gene sequence showed 86.8, 81.4, 81.4, 79.6 and 69.8 % similarity with buffalo, sheep, goat, pig and chicken, respectively. Sequence alignment of the mitochondrial cytochrome B gene differentiated closely related species like cattle and buffalo with divergence score of 14.9 % while sheep and goat showed 13.8 % divergence which enables differentiation even in closely related meat species. Availability of DNA sequences for virtually every species in the centralized databases makes it easier to identify and authenticate the meat species. Thus sequencing and alignment of the mt cyt B sequence using basic local search tool (BLAST) in NCBI database can accurately identify meat species. It has the advantage that this method has high level of accuracy and is applicable for all meat animal species. However, DNA

sequence analysis is costly, time consuming, requires high end equipments and difficult to use for routine species identification tests.

In order to economize and simplify the speciation procedure, another PCR based method, i.e. PCR-RFLP was developed and standardized. Restriction map of nucleotide sequences of mt cyt B gene was analysed for cattle (KC415731), buffalo (KC415732), sheep (KC415729), goat (KC415728), pig (KC 415733) and chicken (KC415730) revealed unique species specific restriction site. Restriction enzyme *MspI* was chosen for PCR-RFLP assay for the differentiation of cattle and pig from other species, as this enzyme generated fragments of 198 and 252 bp in cattle and 389 and 61 bp in pig (Fig. 1b). However, buffalo, sheep, goat and chicken did not possess any restriction site for this enzyme in the targeted sequence. About 35 samples arising from different meat animals (each species) were subjected for PCR amplification and RFLP analysis. Technique was also checked for its applicability in processed meat products. Results revealed similar size of amplicon and restriction products even in the thermally processed beef meat samples heated at 72, 90 and 121°C (Fig. 2a &b). However, the intensity of signal got reduced with the increased cooking temperature and was lowest in those meat samples subjected to 121°C, 15 psi for 30 min. In order to confirm the effectiveness of PCR-RFLP method among different breeds, the procedure was repeated using DNA extracted from blood samples of different cattle breeds of India, viz. Deoni, Red Kandahari and Jersey crossed with local breed and their RFLP patterns are shown in (Fig. 4) which reveals applicability of the technique in different breeds of cattle. RFLP was helpful in showing diagnostic polymorphism created by point mutations which were highly conserved within mt cyt B gene. Restriction profiles obtained were very valuable as genetic markers to authenticate the presence of beef.

To study the efficacy of the technique to detect the extent of adulteration of beef in buffalo meat, similar studies were conducted on mixed meats containing various combinations of beef in buffalo meat i.e. 5:95, 10:90, 20:80, 40:60, 50:50, 60:40, 80:20, 90:10, 95:5. The *MspI* restriction enzyme digested fragments of beef (198+252 bp) were clearly visible in 50, 40, 20 and 10 percent presence of beef in buffalo meat but was not clearly visible at 5% level. Beef

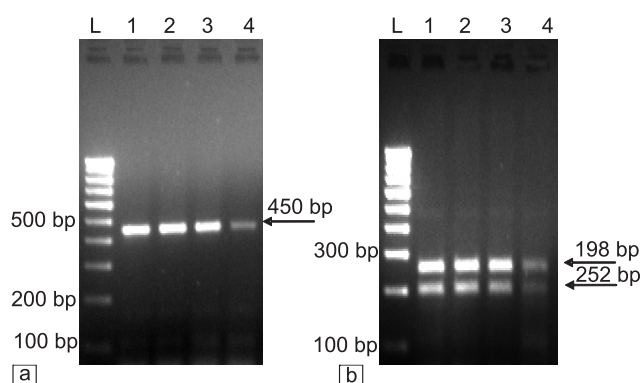
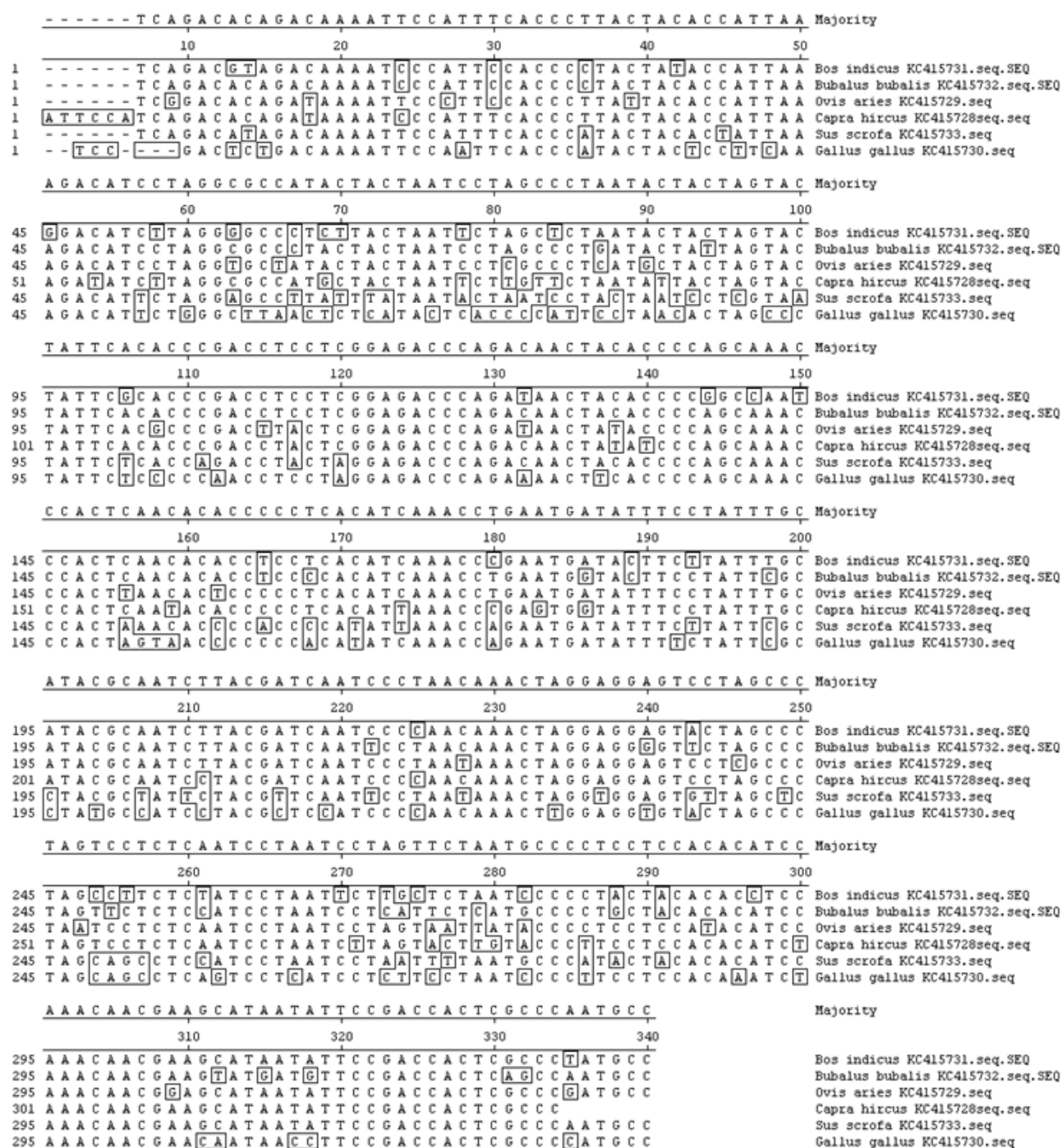


Fig. 2. (a) PCR amplification of mt cytochrome B gene in thermally processed beef samples; 2 (b): PCR-RFLP of mt cytochrome B gene with *MspI* restriction enzyme in thermally processed beef; Lane L: 100 bp ladder; 1: Fresh beef; 2: Meat roasted at 72°C for 30 min; 3: Meat steam cooked at 90°C for 30 min; 4: Meat autoclaved at 121°C, 15 psi for 30 min.



Decoration 'Decoration #1': Box residues that differ from the Consensus.

Fig. 3. Aligned mitochondrial cytochrome B gene nucleotide sequences of different meat animals.

detection was taken as possible up to 10% admixture of beef in buffalo meat (Fig. 5). Although the intensity of bands of beef was low at 10% meat mixture, RFLP pattern was clearly detectable on the 2% agarose gel electrophoresis. Similar result in heat treated meat products was reported by Murugaiah *et al.* (2009).

Nucleotide sequencing is a highly accurate technique which not only checks the authenticity of labeling but will also determine the species to which meat belongs to.

However, method is costly as sequencing requires sophisticated equipments. In addition, sequencing cannot be used for admixed samples. Whereas, RFLP based method is simple, rapid and versatile. Method is economical and does not require sophisticated equipments (Boonphakdee and Sawangwong 2008). However, RFLP results may be affected by intra-specific polymorphism and mutation at the restriction site. In the cases involving legal issues which require very high accuracy combination of RFLP and

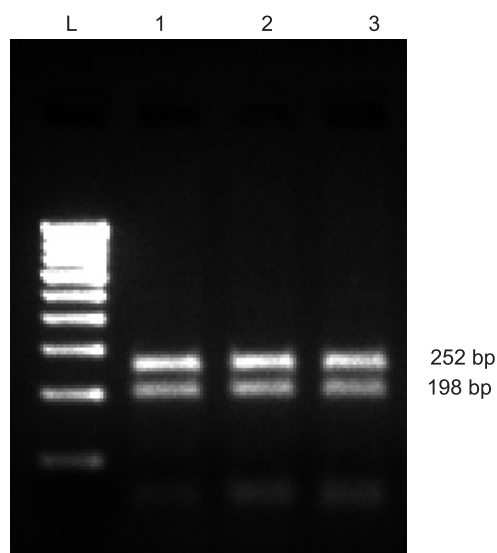


Fig. 4. PCR - RFLP of mitochondrial cytochrome B gene with *MspI* restriction enzyme in different cattle (*Bos indicus*) breeds; Lane L: 100 bp ladder; 1: Deoni breed; 2: Red Kandhari breed; 3: Jersey crossed with local breed

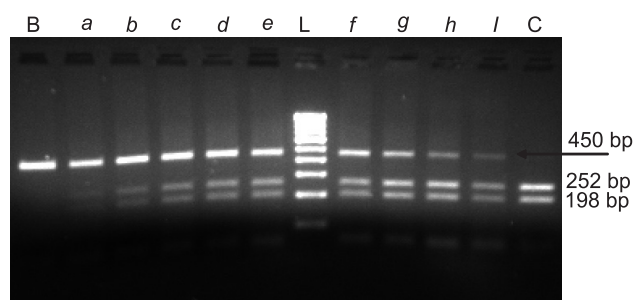


Fig. 5. Polymerase chain reaction - restriction fragment length polymorphism of mt Cytochrome B gene in admixed meats (beef in carabeef). Percent of beef in buffalo meat mixtures digested with *MspI* restriction enzyme (a: 5:95; b: 10:90; c: 20:80; d: 40:60; e: 50:50; f: 60:40; g: 80:20; h: 90:10; i: 95:5); Lanes L: 100 bp ladder; C: 100% beef; B: 100 % carabeef.

sequencing method can conclusively identify the species origin of meat.

Combination of nucleotide sequence analysis and PCR-RFLP techniques targeting mitochondrial cytochrome B gene can be test of choice for the detection of origin of meat and authentication beef and pork. Sequence analysis albeit costly was found suitable for the identification of all species under study while, the RFLP technique was found specific and sensitive for the accurate and precise authentication of beef and pork in raw, cooked and adulterated meat samples. In view of the social, religious, economic, forensic and public health issues relating to animal species authentication, the present work would

enable the analysts/ laboratories to solve the problem of animal species authentication.

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