Title: Molecular evidence for zoonotic transmission of *Giardia duodenalis* among dairy farm workers in West Bengal, India

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Abstract

No study in the past has examined the genetic diversity and zoonotic potential of *Giardia duodenalis* in dairy cattle in India. To assess the importance of these animals as a source of human *G. duodenalis* infections and determine the epidemiology of bovine giardiasis in India, fecal samples from 180 calves, heifers and adults and 51 dairy farm workers on two dairy farms in West Bengal, India were genotyped by PCR-RFLP analysis of the β-giardin gene of *G. duodenalis* followed by DNA sequencing of the PCR products. Phylogenetic analysis was carried out on the DNA sequences obtained in the study and those available in GenBank. The overall prevalence of *G. duodenalis* in cattle was 12.2% (22/180), the infection being more prevalent in younger calves than in adult cattle. Zoonotic *G. duodenalis* Assemblage A1 was identified in both calves and workers although the most prevalent genotype detected in cattle was a novel Assemblage E subgenotype. These findings clearly suggest that there is a potential risk of zoonotic transmission of *G. duodenalis* infections between cattle and humans on dairy farms in India.

Keywords: Giardia duodenalis, Cattle, dairy farm workers, zoonoses, India, Genotyping.

1. Introduction

Protozoan parasites of the genus *Giardia* have a worldwide distribution and are emerging as one of the most frequent causes of diarrhoea in humans in both the developing and developed world. Giardiasis contributes to diarrhoea and nutritional deficiencies in children less than 10 years of age with the highest prevalence in developing countries (Islam, 1990). In addition to humans, they infect a wide variety of domesticated and wild animals having emerged as important parasites of dairy cattle because of their proven pathogenicity (Xiao *et al.*, 1993; Ruest *et al.*, 1997; O'Handley *et al.*, 1999), and the potential public health significance of zoonotic transmission (Buret *et al.*, 1990; Ey *et al.*, 1997; Olson *et al.*, 2004). *Giardia duodenalis* (syn *G. intestinalis*, *G. lamblia*) has been implicated as an etiological agent in dairy and beef calf diarrhoea, worldwide (O'Handley *et al.*, 1999; Huetink *et al.*, 2001; Olson *et al.*, 2004). In fact, reduced rate of weight gain, impaired feed efficiency and decreased carcass weight were associated with giardiasis in a ruminant model of the disease (Olson *et al.*, 1995).

G. duodenalis is the only species found in humans, although it is also found in other mammals, including pets and livestock (Thompson *et al.*, 2000). Substantial evidence suggests *G. duodenalis* to be a species complex comprised of morphologically indistinguishable isolates which can genetically be differentiated into several major assemblages: Assemblages A and B mainly infect humans but are also found in a wide range of other mammals; C and D have been found to infect dogs; E has been isolated from livestock (cattle, sheep, and pigs); F and G have been reported from felines and rats respectively (Monis *et al.*, 2003). More recently, Assemblage H has been detected in marine vertebrates (Lasek-Nesselquist *et al.*, 2010). Recent studies throughout the world have demonstrated that calves in dairy and beef herds may harbour more than one genotypes of *G. duodenalis* (Trout *et al.*, 2004, 2005; Itagaki *et al.*, 2005; Lalle *et al.*, 2005; Mendonca *et al.*, 2007; Langkjaer *et al.*, 2007; Winkworth *et al.*, 2008). However, although the World Health

Organization has considered *G. duodenalis* to have a zoonotic potential for around 30 years (WHO, 1979), direct evidence has been lacking.

A significant prevalence of *Giardia* isolates has been earlier reported in Indian dairy cattle based on microscopic findings (Deshpande and Shastri, 1981). However, surprisingly, there have been no prior molecular characterization studies of *Giardia* in Indian cattle although extensive molecular epidemiological studies have been carried out in a number of countries. There is also lack of data required for the assessment of zoonotic transmission of *G. duodenalis* between cattle and humans in India. Consequently, this study has been formulated to understand the public health significance of this parasite from cattle and get a clearer epidemiological picture, with better information on the zoonotic potential and transmission mechanisms for humans.

2. Materials and Methods

2.1. Collection of fecal samples

Bovine fecal samples used in this study were collected from 180 dairy cattle including 40 pre-weaned calves (0-2 months old), 72 post-weaned calves (3-12 months old) and 68 heifers and adults (> 12 months) with and without diarrhoea from two dairy farms: the Harringhata Cattle Farm, Nadia and Ramakrishna Mission Dairy Farm, Narendrapur, 24 Parganas (N), West Bengal, India from October 2008 to August 2009. Feces were collected directly from the rectum of each animal with a gloved hand and transferred into sterile wide mouthed, labeled plastic containers and immediately placed into an insulated container packed with ice or cold packs. In addition to these, stool samples were also collected from 51 dairy farm workers of these two farms who were in direct or indirect contact with these animals but showed no visible clinical signs of giardiasis. Specimens were transported to the Division of Parasitology, National Institute of Cholera and Enteric Diseases, Kolkata, India as early as possible and processed within two days of collection. Three aliquots of each sample were frozen without preservative in 1.5 ml cryovials at -80 °C for ELISA and PCR studies.

2.2. Parasite Detection

Microscopic examination was performed on all samples within 48 hours after collection. Three separate techniques were used for the detection of *G. duodenalis* in the fecal samples. Firstly iodine wet mount staining and secondly Trichrome stain were performed for the detection of trophozoites and cysts of *Giardia* according to the Centers for Disease Control and Prevention (CDC) method (http://www.dpd.cdc.gov/dpdx/HTML/DiagnosticProcedures.htm). For microscopic screening, parasite cysts and oocysts present in fecal samples were concentrated using a FPC® Fecal Parasite Concentrator (Evergreen Scientific, Los Angeles, CA, USA).

Antigen capture Enzyme Linked Immunosorbent Assay (ELISA) was also performed with all the frozen samples for detection of *Giardia* using a commercially available kit GIARDIA II (TECHLAB, Blacksburg, VA, USA). The monoclonal antibody based ELISA test was used as instructed by the manufacturer.

2.3. DNA extraction

Genomic DNA was extracted from frozen samples from individuals that were positive by microscopy and ELISA using the QIAamp DNA Stool Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions except that the stool lysis temperature was increased to 80 °C. The eluted DNA was quantified spectrophotometrically and stored at -20 °C for further PCR studies.

2.4. PCR Analysis

For the detection of *Giardia*, amplification of a fragment of the β-giardin gene was performed using primers described by Caccio *et al*. (2002) and Lalle *et al*. (2005). A 753 bp fragment was amplified in the primary PCR reaction using the forward primer G7 and reverse primer G759. In the secondary PCR reaction a 511 bp fragment was amplified using the forward primer Glbg511F (5´-GAACGAACGAGATCGAGGTCCG-3´) and the reverse primer Glbg511R (5´-CTCGACGAGCTTCGTGTT-3´). PCR mixtures and cycling conditions were identical to those previously described (Lalle *et al*., 2005) except that the primary PCR reaction mixture also contained non-acetylated BSA (New England Biolabs, Beverly, MA, USA) to a final concentration of 0.1 µg/µl. All PCR products were analysed by 1.5% agarose gel electrophoresis and visualised after ethidium bromide staining.

2.5. Restriction fragment length polymorphism (RFLP) analysis

Secondary PCR products were purified using the High Pure PCR product purification kit (Roche Diagnostics, Mannheim, Germany). The restriction analysis was performed as described before (Lalle *et al*., 2005). Briefly, 10 µl of the nested PCR products of the β-giardin gene were digested with 10 units of restriction endonuclease *Hae*III (New England Biolabs) and 2 µl of 10× buffer in a total volume of 20 µl, at 37 °C for 3 hours. Restriction products were fractionated on a 2% agarose gel and visualised after ethidium bromide staining.

2.6. DNA Sequencing and Phylogenetic Analysis

In order to determine *G. duodenalis* subgenotypes and to confirm the PCR-RFLP results, all purified secondary PCR products that were positive for *Giardia* were directly sequenced in both directions using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA) with forward and reverse primers. Sequences obtained were analyzed and assembled using CLUSTAL W software (Higgins *et al*., 1994). The obtained nucleotide sequences were used to search the GenBank nucleotide sequence database for sequence similarities using BLAST software (NCBI, Bethesda, MD, USA). Multiple alignments of these sequences were made using the BioEdit program (Hall, 1999).

For comparative phylogenetic analysis, reference sequences retrieved from the GenBank were aligned with the representative sequences of each species or genotype of *Giardia* obtained in this study and a neighbor-joining tree was constructed using TREECON for Windows version 1.3b (Van de Peer and De Wachter, 1994). Distance estimations were carried out using the Jukes and Cantor correction. The branch reliability of the neighbor-joining tree was assessed by the bootstrap method with 1000 replications. The nucleotide sequence of *Giardia muris* (GenBank accession no. EF455599) was used as an outgroup to root the neighbor-joining tree since the construction of an unrooted tree showed it to be the most divergent member under analysis.

153 **3. Results**

154 The overall prevalence of *Giardia* in cattle was 12.2% (22/180, Table 1); 27.5% (11/40)
155 pre-weaned calves, 12.5% (9/72) post-weaned calves and 2.9% (2/68) heifers and adults were
156 positive for *Giardia* by both microscopy and ELISA. Among dairy farm workers, *Giardia* was
157 present in 27.4% (14/51) of the screened samples. Successful PCR amplification of the *Giardia* β-
158 giardin gene was accomplished for all the samples positive by microscopy and ELISA.

159

160 *3.1. Genotyping and Phylogenetic Analysis of Giardia*

161 Among cattle, PCR-RFLP results identified *G. duodenalis* Assemblage E as the most
162 prevalent genotype although a substantial number of the isolates from dairy calves were found to be
163 Assemblage A (Table 1). Interestingly, *Giardia* isolates from one pre-weaned and two post-weaned
164 calves produced four visible bands (201, 186, 150 and 110 bp) upon restriction digestion of the β-
165 giardin nested PCR products by *Hae*III (Fig. 1). This meant that there was a mixed *G. duodenalis*
166 Assemblage A and E infection in these calves since RFLP analysis of Assemblage A would have
167 produced three visible bands (201, 150 and 110 bp) while Assemblage E would have generated
168 three bands visible at 186, 150 and 110 bp, upon restriction analysis by *Hae*III (Lalle *et al.*, 2005).
169 DNA sequencing of most Assemblage E positive PCR products (10/14) showed a mismatch of two
170 base pairs with the reference sequences for subtypes E1 (GenBank accession no. AY072729), E2
171 (GenBank accession no. AY545650) and E3 (GenBank accession no. AY653159), the mismatches
172 being at two different positions for each subtype. However, E1 and E2 were also detected in a few
173 samples (Table 2). Additionally, all the Assemblage A positive isolates in cattle were identified as
174 subtype A1 by DNA sequencing of nested PCR products of the β-giardin gene.

175 *G. duodenalis* Assemblage B (57.1%, 8/14) was more prevalent than Assemblage A
176 (42.9%, 6/14) in dairy farm workers (Table 1). DNA sequencing of the nested PCR products

identified A1 and B3 as the dominant subtypes although a number of other subtypes were also found in a few samples (Table 2).

Under phylogenetic analysis, all the *G. duodenalis* assemblages studied (A-F) formed an individual distinct cluster (Fig. 2). Grossly, two major groups were formed. One group comprised of assemblages A, E and F while the other one contained assemblages B, C and D. This indicated that assemblages A, E and F were related to each other and that assemblages B, C and D were related to each other. Intra-Assemblage genetic polymorphism was also evident within assemblages A, B and E.

3.2. Nucleotide sequence accession numbers

Representatives for genotypes of *G. duodenalis* identified in this study have been submitted to GenBank under the accession numbers: GQ290390, GQ345009 and GQ345010.

4. Discussion

There has been considerable interest in recent years in the potential for zoonotic transmission of *G. duodenalis* particularly with respect to cattle and other livestock. Still, until now not even a single study has examined the genetic diversity of *G. duodenalis* in Indian dairy cattle. Also, there is lack of information regarding the zoonotic potential of this parasite for human beings working at dairy farms in a developing country like India. The environment at dairy farms in India is such that close contact of humans with animals occurs regularly, putting dairy farm workers, cattle handlers and veterinarians at risk of contracting zoonotic diseases. This study helps to provide valuable information on both aspects viz. the genetic diversity and the zoonotic potential of *G. duodenalis* from Indian dairy cattle.

The present study represents the first report on the molecular characterization of *G. duodenalis* in Indian cattle. Overall, *Giardia* was detected in 12.2% (22/180) of the bovine fecal samples screened by microscopy, ELISA and PCR studies. The relatively higher prevalence of *Giardia* infection found in a previous study in Indian cattle (Deshpande and Shastri, 1981) can be explained by the fact that only calves were sampled in that study whereas both calves and adult cattle were screened for *Giardia* in the current study. Further, results from the present study indicate that infections by *G. duodenalis* are more prevalent in calves than in adult cattle, which agrees with previous reports (Huetink *et al.*, 2001; Olson *et al.*, 2004; Mendonca *et al.*, 2007). A significant number of Assemblage E isolates detected in calves and adult cattle were shown to be genetically and phylogenically different from all the previous known Assemblage E subtypes namely E1, E2 and E3 (Lalle *et al.*, 2005); however, they were relatively more closely related to E1 and E3 under phylogenetic analysis. This refers a novel subgenotype of *G. duodenalis* Assemblage E in Indian cattle. As expected, this livestock-specific assemblage was found more frequently in cattle sampled in this study, although the most common zoonotic genotype Assemblage A (Thompson *et al.*, 2000) was also identified in a number of calves. Similar observations have been

reported in previous studies from across the globe (O'Handley *et al.*, 2000; Huetink *et al.*, 2001; Trout *et al.*, 2004, 2005; Itagaki *et al.*, 2005; Mendonca *et al.*, 2007; Langkjaer *et al.*, 2007). Additionally, it was also observed that prevalence of Assemblage A was more or less equal in pre-weaned as well as post-weaned calves. Thus no age-related distribution of zoonotic genotypes of *Giardia* was found in this study which is similar to findings from previous studies (Trout *et al.*, 2004, 2005; Langkjaer *et al.*, 2007).

Results of this study provide useful molecular evidence on the zoonotic transmission of *G. duodenalis* between cattle and dairy farm workers. DNA sequencing of nested PCR products of the β-giardin gene genotyped Assemblage A isolates from all calves and most dairy farm workers as subtype A1. It is interesting to note here that the two most common subtypes of Assemblage A, A1 and A2, differ significantly in host preference; animals are mostly infected with A1 whereas humans are mostly infected with A2 (Xiao and Fayer, 2008). Therefore, a relatively higher prevalence of A1 in comparison to A2 found in humans in this study is of zoonotic importance. In addition, mixed *G. duodenalis* Assemblage A and E infections were also detected in three calves in the present study which suggests that calves, although primarily infected with *G. duodenalis* Assemblage E, are frequently hosts of the zoonotic *G. duodenalis* Assemblage A. This is of potential public health significance since calves infected with *Giardia* normally shed a large number of cysts in their faeces (O'Handley *et al.*, 1999) and could thus act as a potential zoonotic reservoir for human giardiasis especially on the dairy farm premises. However, Assemblage B was not detected in cattle in the present study although it was the most prevalent genotype found in dairy farm workers thus indicating anthroponotic transmission of this genotype is more common.

In conclusion, results of this study provide new insights into the epidemiology and genetic diversity of *Giardia duodenalis* in Indian dairy cattle. Additionally, results also point towards a possible zoonotic transmission of this parasite between cattle and human workers on dairy farms in India. Even a few calves infected with zoonotic genotypes of *Giardia* could pose a significant

241 public health risk directly to handlers or indirectly as an important reservoir for human waterborne
242 outbreaks of giardiasis.
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332 two Ohio farms with calf diarrhea. Vet. Parasitol. 51, 41-48.
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335 **Figure Captions**

336 Figure 1. Genotyping of *Giardia* isolates by RFLP analysis based on digestion of β-giardin gene
337 PCR products by *Hae*III. Lanes 1 and 5: 100 bp plus DNA ladders, lane 2: Assemblage E, lane 3:
338 Mixed Assemblage A and E, lane 4: Assemblage A (bovine source), lane 6: Assemblage A (human
339 source) and lane 7: Assemblage B.

340

341 Figure 2. Phylogenetic relationship among *Giardia* isolates as inferred by neighbor-joining analysis
342 of the partial β-giardin nucleotide sequences. Bootstrap values above 50% out of 1000 replicates
343 are indicated at each node. Accession numbers for sequences obtained from GenBank are given in
344 parentheses. The sequence of *Giardia muris* was used as an outgroup.

345

346 **Tables**

347 Table 1. Detection of different genotypes of *Giardia duodenalis* by PCR-RFLP of the β-giardin
348 gene in fecal samples collected from different age groups of dairy cattle and dairy farm workers.

349

Source	Sample size	*Giardia duodenalis*			
		A	B	E	A + E
Pre-weaned calves	40	3	0	7	1
Post-weaned calves	72	2	0	5	2
Heifers and Adult Cattle	68	0	0	2	0
Dairy Farm Workers	51	6	8	0	0

350

351

352 Table 2. *Giardia duodenalis* assemblages A, B and E subtypes identified in dairy cattle and dairy

353 farm workers by DNA sequencing of the β-giardin gene.

354

Host	Cattle		Human	
Assemblage	A	E	A	B
Subtype	A1=5[a]	E_n=10[c]	A1=4	B1=1
	A+E=3[b]	E1=3	A2=2	B3=6
		E2=1		B4=1

355 [a] Number refers to the isolates detected

356 [b] Subtypes could not be determined

357 [c] E_n refers to the novel subtype detected

358

Figure

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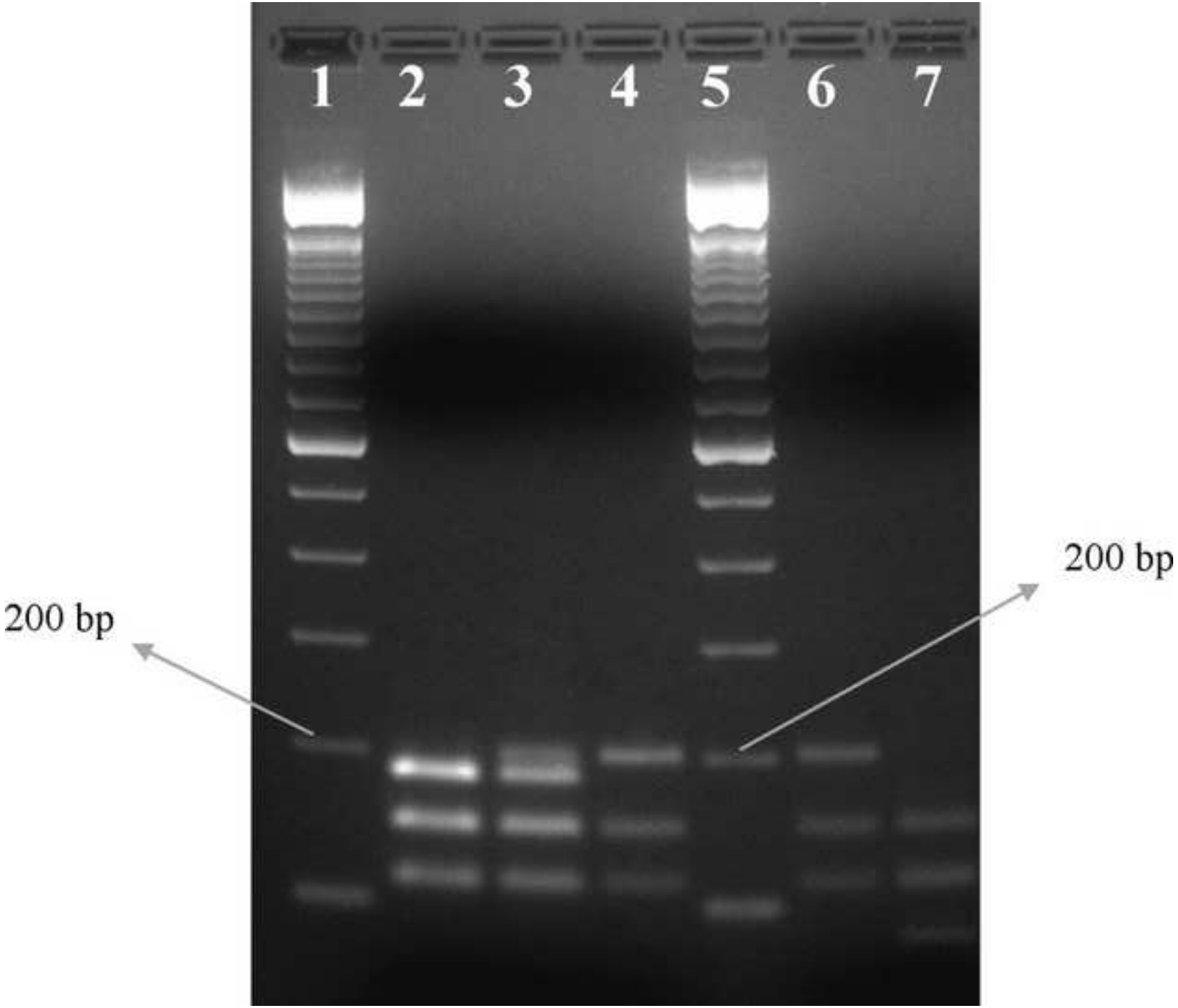

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