

## GROUP-SPECIFIC MULTIPLEX PCR IDENTIFICATION OF CANINE FAECAL *LACTOBACILLUS* SPECIES

<sup>1</sup>N. Mohammad Sharif\*, <sup>1</sup>B. Sreedevi, <sup>1</sup>R.K. Chaitanya, <sup>2</sup>Ch. Srilatha  
and <sup>1</sup>D. Sreenivasulu

<sup>1</sup>Department of Veterinary Microbiology, College of Veterinary Science, Sri Venkateswara  
Veterinary University, Tirupati, Andhra Pradesh -517 502, INDIA.

<sup>2</sup>Department of Veterinary Pathology, College of Veterinary Science, Sri Venkateswara  
Veterinary University, Tirupati, Andhra Pradesh -517 502, INDIA.

E-mail: sharifnoorbasha@gmail.com (\*Corresponding Author)

**Abstract:** A set of forty-nine *Lactobacillus* isolates recovered from the faecal swabs of different dog breeds viz. Pomeranian (12), Labrador (15) and mongrel/indigenous dogs (22) were subjected to *Lactobacillus* group-specific multiplex PCR corresponding to the 16S-23S rRNA intergenic spacer region (ISR) and its flanking 23S rRNA gene. Twenty-three (46.9%) isolates were found to be positive for Group IV; six (12.2%) isolates for Group I and four (8.1%) isolates for Group II. In our study, sixteen (32.6%) isolates could not be identified with the used primers and hence were not assigned into any group. None of the isolates were found positive for the phylogenetic Group III. Amplification of 16S-23S rRNA ISR and its flanking 23S rRNA gene in the present study identified 33 canine faecal *Lactobacillus* isolates into three phylogenetic groups.

**Keywords:** Dogs, group-specific multiplex PCR, *Lactobacillus*.

### Introduction

Lactic acid bacteria (LAB) of the genus *Lactobacillus* are the most important probiotic organisms used in canine medicine to treat and prevent various clinical gastrointestinal disturbances (Baillon *et al.*, 2004). Despite the increasing interest on probiotics as a therapeutic tool, there are only a few studies focused on probiotic organisms of canine origin. To date, most probiotics for pet use are strains isolated from humans or farm animals (McCoy and Gilliland, 2007). Successful canine probiotic organism would ideally be derived from the canine gastrointestinal tract, as they exert host-specific effects (McCoy and Gilliland, 2007).

Traditional identification of LAB is based phenotypic characteristics like morphology, fermentation of various carbohydrates, mode of fermentation and growth at different temperatures (Holzapfel *et al.*, 2001). Phenotypic identification is not always reliable in discriminating the closely related species (Schleifer *et al.*, 1995). Consequently, PCR-based methods using oligonucleotides corresponding to 16S rRNA gene or 16S-23S rRNA intergenic spacer region (ISR) and its flanking 23S rRNA gene have been widely used for the

specific identification and detection of probiotic strains (Dubernet *et al.*, 2002 and Song *et al.*, 2000). Proper identification and classification of *Lactobacillus* species gives a strong indication of its technical applicability for use in probiotic products. The present study aimed at the identification of a set of forty-nine *Lactobacillus* isolates recovered from the faecal swabs of dogs using group-specific multiplex PCR targeting 16S-23S rRNA intergenic spacer region (ISR) and its flanking 23S rRNA gene.

### Materials and methods

**Reference strains:** The strains used in this study, *Lactobacillus acidophilus* (MTCC 10307), *L. plantarum* (MTCC 9496), *L. delbrueckii* sub-species *lactis* (MTCC 911), and *L. rhamnosus* (MTCC 1408) were obtained from Microbial Type Culture Collection and Gene Bank, Chandigarh (India). The choice of lactobacilli strain was carried out according to the four phylogenetic groups as described by Song *et al.* (2000).

**Source of bacterial isolates:** Forty-nine *Lactobacillus* isolates recovered from the faecal swabs of different dog breeds viz. Pomeranian (12), Labrador (15) and mongrel/indigenous dogs (22) were used in the present study. Isolation and identification of isolates was done as per standard cultural and biochemical tests of *Lactobacillus* species using MRS (de Man, Rogosa and Sharpe) medium (Sneath and Holt, 2001). DNA isolation was carried out by boiling and snap chilling method (Wani *et al.*, 2006).

**Oligonucleotide primers:** A set of four forward primers (LU 1F, 5'-ATT GTA GAG CGA CCG AGA AG-3'; LU 3F, 5'-AAA CCG AGA ACA CCG CGT T-3'; LU 5F, 5'-CTA GCG GGT GCG ACT TTG TT-3' and Ldel 7F, 5'-ACA GAT GGA TGG AGA GCA GA-3') and one reverse primer (Lac 2R, 5'-CCT CTT CGC TCG CCG CTA CT-3') corresponding to 16S-23S rRNA intergenic spacer region (ISR) and its flanking 23S rRNA gene were used in the present study as described by Song *et al.* (2000).

**Thermal cycling conditions:** *Lactobacillus* isolates were subjected to multiplex PCR for grouping into four taxonomic groups (I, II, III and IV) based on phylogeny derived from 16S-23S rRNA intergenic spacer region (ISR) and its flanking 23S rRNA gene (Song *et al.*, 2000). Amplification was optimized in 25 µl reaction mixture (containing 2.0 µl of DNA template prepared from each isolate; *Taq* buffer [10x] – 2.5 µl; dNTP mix [10mM] – 1.0 µl; MgCl<sub>2</sub> [25mM] - 1.0 µl; forward primer [10 pmol/µl] – 0.4 µl each; reverse primer [10 pmol/µl] – 1.6 µl; *Taq* DNA polymerase [1 U/µl] - 1 µl and nuclease free water – 14.3 µl). DNA fragments were amplified as follows: initial denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 1 min, elongation at 72°C for 1 min

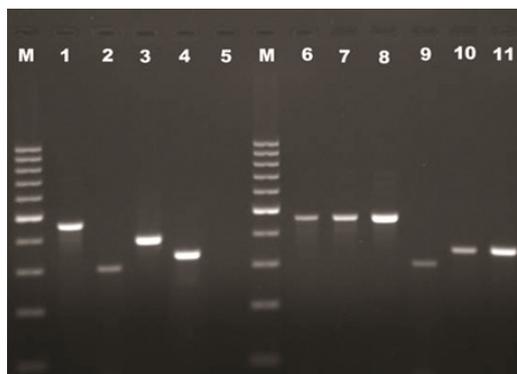
and final elongation at 72 °C for 7 min. The expected sizes of amplicons were as follows: 450 bp (group I), 300 bp (group II), 400 bp (group III), and 350 bp (group IV). Reference strains *L. delbrueckii* MTCC 911 (Group I); *L. acidophilus* MTCC 10307 (Group II); *L. rhamnosus* MTCC 1408 (Group III); *L. plantarum* MTCC 9496 (Group IV) served as positive controls.

### Results and discussion

Out of 49 isolates, amplification with primers targeting 16S-23S rRNA intergenic spacer region (ISR) and its flanking 23S rRNA gene generated a PCR product for 33 isolates. A total of 23 (46.9%) isolates were found to be positive for Group IV; 6 (12.2%) isolates for Group I and 4 (8.1%) isolates for Group II, whereas 16 (32.6%) isolates were found to be negative and could not be assigned into any group. None of the isolates were found positive for the phylogenetic Group III (Table 1 and Fig. 1).

**Table 1: Multiplex PCR grouping of canine faecal *Lactobacillus* isolates**

S.No	Dog breed	Number of <i>Lactobacillus</i> isolates			
		<i>Lactobacillus</i> group-specific multiplex PCR			
		Group I	Group II	Group IV	Unassigned
1.	Mongrel	4	2	11	5
2.	Labrador	2	1	5	7
3.	Pomeranian	0	1	7	4
	<b>TOTAL</b>	<b>6</b>	<b>4</b>	<b>23</b>	<b>16</b>



**Fig. 1: Gel photograph of amplicons of *Lactobacillus* group-specific multiplex PCR. Lane M: Molecular weight marker (100 bp), Lane 1: DNA standard of *L. delbrueckii*, MTCC 911 (Group I, 450 bp), Lane 2: DNA standard of *L. acidophilus*, MTCC 10307 (Group II, 300 bp), Lane 3: DNA standard of *L. rhamnosus*, MTCC 1408 (Group III, 300 bp), Lane 4: DNA standard of *L. plantarum*, MTCC 9496 (Group IV, 350 bp), Lane 5: Negative control, Lane 6-8: Dog faecal *Lactobacillus* isolates positive for Group I (450 bp), Lane 9: Dog faecal *Lactobacillus* isolate positive for Group II (300 bp), Lane 10 and 11: Dog faecal *Lactobacillus* isolates positive for Group III (400 bp).**

In a study by Song *et al.* (2000) in Japan, phylogenetic analysis of 17 *Lactobacillus* strains based on nucleotide sequence of the 16S-23S rRNA intergenic spacer region (ISR) revealed four distinct groups i.e. Group I (*L. delbrueckii subsp. bulgaricus* and *subsp. lactis*), group II (*L. acidophilus*, *L. amylovorus*, *L. crispatus*, *L. helveticus*, *L. gasseri* and *L. johnsonii*), group III (*L. casei*, *L. paracasei* and *L. rhamnosus*) and group IV (*L. fermentum*, *L. animalis*, *L. plantarum*, *L. reuteri*, *L. ruminis*, *L. salivarius* and *L. vaginalis*). In the case of closely related species, primers targeting 16S rRNA gene alone cannot be used due to little variation in the 16S rRNA sequence, which is conserved among other genera of lactic acid bacteria like *Pediococcus* and *Weissella* (Schleifer *et al.*, 1995). Instead, the sequence of the 16S-23S rRNA intergenic spacer region (ISR) was more suitable for designing specific primers in the identification of closely related species as it exhibits greater variations than that of the 16 rRNA structural gene (Barry *et al.*, 1991). Group-specific primers targeting 16S-23S rRNA intergenic spacer region (ISR) were successfully employed earlier by Garg *et al.* (2009) for the identification of spectrum of *Lactobacillus* species in the vagina of healthy women in India.

In summary, group-specific multiplex PCR assay of the present study identified 33 out of 49 *Lactobacillus* isolates screened into three different phylogenetic groups. A total of 16 isolates that had shown morphological and biochemical characteristics in accordance with the genus *Lactobacillus* didn't amplified the 16S-23S rRNA intergenic spacer region (ISR) and its flanking 23S rRNA gene targeted in the present study.

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