

MOLECULAR CHARACTERISATION AND PHYLOGENETIC ANALYSIS OF PORCINE BETA-DEFENSIN-1 GENE IN ANKAMALI PIGS OF INDIA

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Abstract: Ankamali pigs from Kerala possess unique qualities such as disease resistance and adaptation to the local environment. Porcine beta defensin-1 (PBD-1) gene plays an important role in the innate immunity of pigs by encoding an antimicrobial peptide that is active against several bacteria and fungi. Present study was undertaken to characterise the PBD-1 gene in Ankamali pigs. Total RNA was extracted from the tongue of Ankamali pigs and cDNA were synthesized by Reverse Transcription –Polymerase Chain Reaction (RT-PCR). The PBD-1 gene was amplified from the cDNA and sequenced. Nucleotide sequencing revealed that the product was exactly 243 bp long encompassing the 192 bp long complete coding sequence (CDS) of PBD-1 gene. The gene encoded a peptide of 64 amino acids. The PBD-1 sequence from Ankamali showed complete homology with that of other pigs. Phylogenetic tree constructed from homologous sequences illustrated the evolutionary relationship of PBD-1 with beta-defensins of other species.

Keywords: Ankamali; Beta-defensins; Molecular Characterisation; Phylogenetic Analysis.

INTRODUCTION

Use of antibiotics has become very common in livestock practice and it is a common observation that recommended withdrawal period is often ignored. This results in presence of residues in milk and meat. In addition, the use of antibiotics at suboptimal doses results in emergence of resistant strains of pathogens. Thus, there is a need to develop an alternate approach that would lessen our dependence on antibiotics. Antimicrobial peptides (AMPs) are an important part of mammalian innate immune system. They are shown to be potent against microbes, possess low haemolytic activity and have high thermal stability even at 80 °C (Jiang *et al.*, 2006; Li *et al.*, 2013). Characterisation of genes encoding these molecules might provide a template for synthesis of novel peptide antibiotics in future.

AMPs are polypeptides made up of 100 or fewer amino acid residues and act against a wide range of microbes (Ganz, 2003). Based on the net charge present, AMPs are broadly classified into anionic and cationic peptides (Hancock, 1997). Among cationic AMPs,

defensins are a subclass with broad spectrum antimicrobial activity against various bacteria, fungi and viruses. Based on intramolecular disulphide bonds between highly conserved cysteine residues, three families of defensins are defined *viz.* α -, β - and θ -defensins (Lai & Gallo, 2009). The porcine β -defensin-1 (PBD-1) peptide found at higher concentrations in the oral cavity of pigs has direct action against several bacteria and fungi and, thus plays a vital role in the innate immunity of pigs (Zhang *et al.*, 1999; Shi *et al.*, 1999; Li *et al.*, 2013).

Indian native pigs have unique qualities such as disease resistance, adaptation to the local environment and possession of lean fat (De *et al.*, 2013). Coding sequences of antimicrobial peptide genes from important livestock species have been sequenced and characterised *viz.* cattle (Schonwetter *et al.*, 1995), buffalo (Kalita and Kumar, 2009), sheep (Huttner *et al.*, 1998) and goat (Zhao *et al.*, 1999). However, to date, antimicrobial peptide gene from oral cavity of indigenous pigs has not been characterised. Therefore, the present investigation was undertaken to characterise the complete coding sequence (CDS) of PBD-1 gene in Ankamali pigs. In addition, the PBD-1 sequence obtained in the present study was compared with beta-defensin-1 sequences of 16 other species available in the NCBI database.

MATERIALS AND METHODS

Collection of tissue samples

Tongue tissue samples were collected from adult Ankamali pigs reared at Centre for Pig Production and Research, Mannuthy, Thrissur, Kerala, India immediately after the slaughter of the animal. Sections of the tissues (~200 mg) were taken using a sterile Baird-Parker (BP) blade with gloved hands and were immersed quickly into 1 ml RNA *later* solution (Sigma Aldrich, USA).

Extraction of RNA

About 100 mg of RNA *later* stabilised tissue was used for isolation of total RNA using 1 ml TRI reagent (Sigma Aldrich, USA) as per manufacturer's instructions. Tissue was homogenised for 90 seconds with the help of a polytron homogeniser (IKA T10 basic ULTRA-TURRAX Homogenizer system, Germany). The RNA was quantified in NanoDrop™ 2000c spectrophotometer (Thermo Scientific, USA) and its integrity was assessed by 1.2% denaturing agarose gel electrophoresis.

DNase treatment and cDNA synthesis

Genomic DNA contamination, if any, was removed by Deoxyribonuclease (DNase) treatment of RNA samples with a commercially available DNase (DNase I, Amplification Grade, Sigma Aldrich, USA). Complementary DNA (cDNA) was synthesised from 1 μ g of DNase

treated RNA using Revert Aid first strand cDNA synthesis kit (Thermo Scientific, USA). Random hexamer primers were used for amplification. Resultant cDNA was stored at -20 °C until further use.

Amplification of PBD-1 gene

The complete coding sequence (CDS) of PBD-1 gene was amplified using cDNA as template. Published primers used in the study were: Forward 5'-ACCAGCATGAGACTCCACC-3' and reverse 5'-GCTTCTGAGCCATATCTGTG-3' (Qi *et al.*, 2009). PCR was carried out in a thermal cycler (Bio-Rad, USA). Each 50 µl volume reaction contained 1X Taq polymerase buffer, 1.5 mM MgCl₂, 0.2 mM of dNTP mixture, 10 pM/µl each of forward and reverse primers, 1.5 units of Taq polymerase and nuclease free water to make up the volume. The PCR conditions followed were initial denaturation for 3 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 20 sec at 58.8°C, 30 sec at 72°C and final extension at 72°C for 8 min. The amplified PCR products were resolved in 2% (w/v) agarose gel in 1X TBE.

Nucleotide sequencing and analysis

PCR products obtained were sequenced using respective forward and reverse primers in an automated sequencer (ABI prism, USA) using Sanger's dideoxy chain termination method (Sanger *et al.*, 1977) at SciGenom Labs Pvt. Ltd., Cochin. The sequences obtained were analysed using the 'MegAlign' tool of Lasergene Software (DNASTAR, USA) to generate sequence alignment reports, sequence distances and phylogenetic trees.

RESULTS AND DISCUSSION

Concentration, Purity and Quality of RNA

The concentration of total RNA extracted from tongue epithelium of Ankamali pigs was 506.38 ng/µl. The Optical Density (OD) ratio (260:280) of extracted RNA was 2.06. The integrity of extracted RNA was verified by agarose gel electrophoresis (Figure 1a). The 28S and 18S rRNA bands were clear and intensity of the 28S rRNA band was almost twice that of the 18S rRNA indicating good quality RNA. The mRNA was observed as smear spanning between 28S and 18S rRNA. The absence of band near the well indicated the purity of RNA sample from genomic DNA contamination. The PCR product at optimum annealing temperature (58.8°C) yielded a specific product of 243 bp upon 2 % agarose gel electrophoresis (Figure 1b).

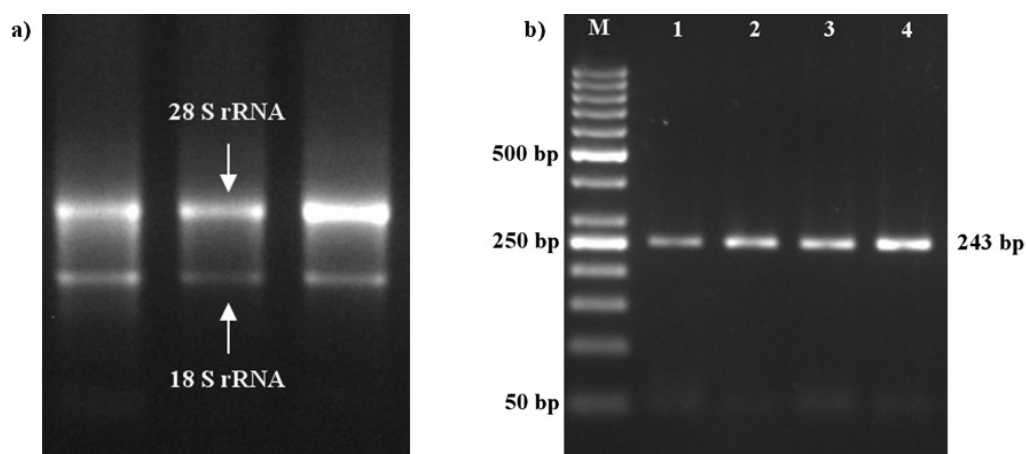


Figure 1. a) Total RNA isolated from different tissues of Ankamali and LWY pigs. b) PCR amplification of 243 bp fragment of PBD-1 gene from cDNA. Lane 1-4: 243 bp PCR product of PBD-1 gene. Lane M: 50 bp DNA marker.

Sequence analysis

Sequencing of amplified PCR product revealed that the product was exactly 243 bp long encompassing the 192 bp complete CDS of PBD-1 gene. The sequence obtained in the present study from Ankamali pigs was submitted to the NCBI database (Accession No. KP862533). The 192 bp long complete CDS of PBD-1 gene obtained from Ankamali pig showed 100 per cent identity with that of other pigs. This indicates that the sequence of PBD-1 in Ankamali pigs has not changed in spite of its stressful tropical habitat. Similar results were reported in case of Tibetan pigs by Qi *et al.* (2009). This shows that PBD-1 gene is highly conserved in different breeds of pigs irrespective of their habitats and thus it might be playing an important role along with many other molecules in the adaptation of animals to different environments. The “Protean” tool predicted a low molecular weight (7.07 kDa) and high isoelectric point (10.24) for the deduced PBD-1 peptide. Low molecular weight for the recombinant PBD-1 peptide has been reported by other researchers (Li *et al.*, 2013). There were no negatively charged (acidic) amino acids in the deduced peptide sequence whereas the number of basic amino acids was found to be 12. In addition, the peptide was found to have positive charge at pH 7. This confirms the cationicity of PBD-1 peptide which is an essential feature of cationic AMPs to act on the cell membrane of microbes. These findings are in accordance with the conventional description of cationic AMPs given by Hancock and Diamond (1998).

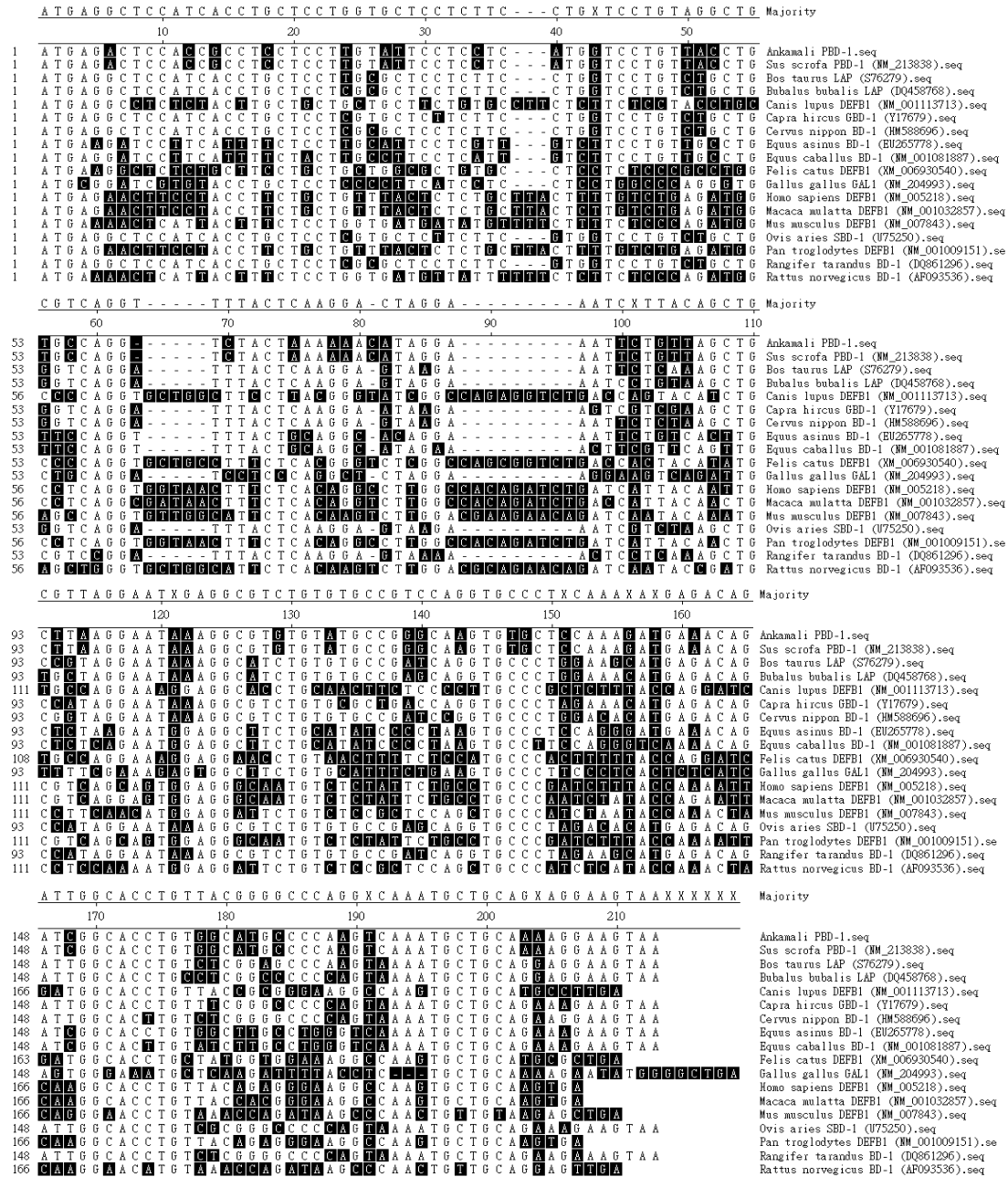


Figure 2. Nucleotide sequence alignment report of beta-defensin-1 genes of different species. Shaded portion indicates residues that differ from the consensus

Nucleotide Sequence homology

The complete CDS of PBD-1 gene obtained from Ankanamali pig was subjected to multiple sequence alignment with PBD-1 of other pig and beta-defensin-1 sequences of 16 other species (Figure 2). Sequences were retrieved from the NCBI GenBank. The species considered for comparison included six artiodactyles (cattle, buffalo, sheep, goat, spotted deer and reindeer), two perissodactyles (horse and donkey), three primates (human, chimpanzee and monkey), two carnivores (cat and dog), two rodents (rat and mouse) and a

bird (chicken). Upon subjecting nucleotide sequences to multiple sequence alignment, the PBD-1 sequence from Ankamali showed relatively higher per cent identity with sheep (75.8), buffalo (75.3), cattle (74.7), spotted deer (74.7), goat (73.7), reindeer (72.7), donkey (71.6) and horse (66) compared to mouse (46.4), chimpanzee (46), human (45.5), chicken (45.5), rat (45.3), monkey (45), dog (43.2) and cat (40.6). Same pattern was noticed in divergence analysis which showed highest divergence of PBD-1 sequence from the sequence of cat and the least divergence from that of other pigs. These findings suggest that the β -defensin locus is conserved in even-toed ungulates and are in accordance with the findings of Zhang *et al.* (1998).

Phylogenetic analysis

The phylogenetic trees (Figure 3) were constructed to estimate the evolutionary relationship among the β -defensins of different mammals using “MegAlign” programme of Lasergene software. Nucleic acid sequences from 16 different species were compared with that of PBD-1. Reliability of the tree was tested with the help of bootstrap analysis. Hall (2013) noticed that the bootstrap test estimates the reliability of each node instead of reliability of the whole tree. It was also suggested that the nodes with < 70 per cent bootstrap value may not be informative.

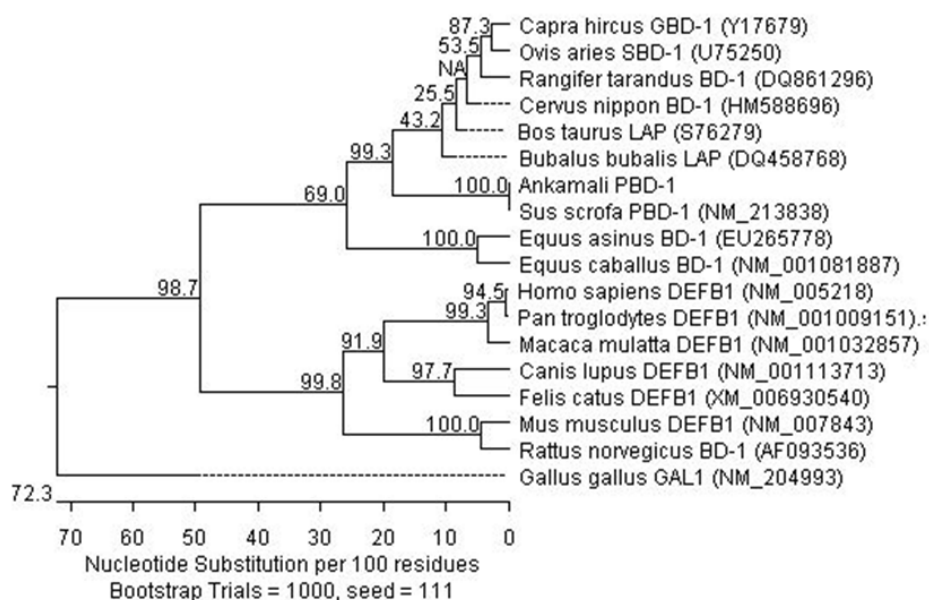


Figure 3. Phylogenetic tree based on the nucleotide sequence of PBD-1 and other species

All the major branches had a bootstrap value ranging from 70 to 100 per cent indicating its better reliability. The nodes within the ruminantia group were quite unreliable with low

bootstrap values. Two unique branches were formed for birds and mammals in the phylogenetic tree constructed from β -defensin-1 nucleotide sequences of different species. Among mammals, two major branches were formed between ungulates (ruminant, swine and equine) and other mammalian species (primates, carnivores and rodents). Within the ungulates, perissodactyles and artiodactyles formed sub branches. Pigs formed a separate group from other artiodactyles. Sang *et al.* (2006) had reported that PBD-1 formed a separate branch in a phylogenetic tree constructed for human and porcine β -defensin genes. Choi *et al.* (2012) had observed a close relationship between PBD-1 and human β -defensin-4 (HBD-4) in phylogenetic analysis on β -defensin genes of human, pigs and cattle. It may be inferred from above results that among the different species compared, the PBD-1 gene was quite different from other species in terms of structure, organisation and composition.

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