RESEARCH ARTICLE
JIVA 22 (2) August 2024
https://doi.org/10.55296/JIVA/22.2.2024.35-41

Received on 11/07/2024
Accepted on 24/07/2024

IDENTIFICATION OF GENOME-WIDE VARIANTS IN ANKAMALI PIGS OF KERALA BY WHOLE GENOME SEQUENCING

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ABSTRACT

Ankamali pigs are a domesticated indigenous pig variety from Kerala, recognised for their resistance to diseases, production of lean meat and ability to thrive in tropical humid climates. However, there is still limited understanding of Ankamali pig genome variation, genetic relationships with other pig breeds and the process of domestication. Here, we focus on elucidating the genome-wide variants in Ankamali pig through whole genome sequencing. Whole genome sequencing of the Ankamali pig genome generated 205.66 Gb of raw data. By GATK Haplotype Caller algorithm we identified a total of 26.6 million single nucleotide variants (SNVs) including 21.3 million single nucleotide polymorphisms (SNPs) and 5.3 million InDels. Out of the total number of SNPs obtained, 69.59 per cent were transitions and 30.41 per cent were transversions. During functional annotation of SNVs, 66.19 per cent of the mutations were silent, 33.53 per cent were missense and 0.29 per cent were nonsense mutations. The potential variants identified in this study can facilitate future research into the positive attributes of Ankamali pigs, thereby aiding in the development of more effective conservation strategies.

Keywords: Ankamali pig, Whole genome sequencing, Variant calling, single nucleotide variants

INTRODUCTION

According to the United Nations Food and Agriculture Organisation, pork is the most widely consumed meat in the world, accounting for 36 per cent of global meat consumption. In India, as of the 20th livestock census in 2019, the pig population was estimated at 9.06 million. However, the number of indigenous pigs is declining due to extensive crossbreeding

with exotic germplasm. Indigenous pig population comprises a small number of recognised breeds distinguished by specific breed characteristics, while the majority are classified as non-descriptive or desi pigs. Among these, the Ankamali pig is a domesticated variety that is primarily raised in Kerala. Although Ankamali pigs exhibit slower growth rates and less efficient feed conversion ratios compared to exotic or crossbred pigs (Gaur *et al*., 1997), they possess distinct advantages such as disease resistance, heat tolerance and the ability to produce leaner meat in comparison to exotic breeds (Behl *et al*., 2006). This leanness makes the meat of Ankamali pigs preferred in the market, commanding higher prices than meat from exotic or crossbred breeds. To establish a cost-effective conservation strategy, it is essential to assess the genetic makeup of Ankamali pig variety. This helps in identifying and conserving distinct genetic variations within Ankamali pigs for conservation purposes, ensuring that maximum genetic diversity is maintained at minimal expense.

Advancements in genetics and genomic technologies have led to the discovery of more genes and genomes (Enard *et al*., 2014; Frantz *et al*., 2015). Additionally, as sequencing costs have decreased, powerful tools are now accessible for studying these genetic elements. In the past decade, the adoption of high throughput SNP genotyping and whole-genome sequencing has offered detailed insights into genome-wide variants in various livestock species (Rubin *et al*., 2012; Zhao *et al*., 2018). The aim of this study was to identify the variants from whole genome sequence data of Ankamali pigs using variant calling techniques.

MATERIALS AND METHODS

Experimental animals and whole-genome sequencing

Blood samples were collected from 12 Ankamali pigs (6 females and 6 males) reared under uniform management conditions and fed with pelleted pig feed at the Centre for Pig

Production and Research, Kerala Veterinary and Animal Sciences University (KVASU), Mannuthy,. DNA samples were extracted from all pigs using the Qiagen DNeasy Tissue kit (Qiagen, Düsseldorf, Germany), and the integrity and purity of the DNA were verified by agarose gel electrophoresis and A260/280 ratio. The DNA samples from the 12 Ankamali pigs were pooled together at equimolar concentrations and subjected to high-throughput sequencing by outsourcing (Clevergene, Bengaluru). The whole genome sequencing library was prepared using the QIAseq FX DNA Library Kit for Illumina, followed by end-repair, A-tailing,

ligation of pair-ended adapters, size-selection for sequencing and amplification. Finally, the amplified library was analysed and loaded onto the Illumina NovaSeq 6000 platform for cluster generation, followed by paired-end sequencing.

Reads alignment, variant calling and annotation

To ensure high-quality data, a Fastq quality check was conducted to analyse parameters such as base quality score distribution, average base content per read and GC distribution in the raw sequence data. Reads that passed the quality check were aligned to the Sus scrofa reference genome (Suss11.1, GenBank assembly GCF_000003025.6) using BWA- MEM (Li and Durbin, 2009). The resulting bam file was sorted using Picard SortSam and processed to remove duplicates using Picard MarkDuplicates (http://picard.sourceforge.net). Subsequently, the GATK HaplotypeCaller algorithm (McKenna *et al*., 2010) was applied to call variants in the Ankamali pig genome. The identified variations were classified into types such as single nucleotide variants (SNVs), single nucleotide polymorphisms (SNPs), insertions (INS), deletions (DEL), among others. The count of transitions and transversions was determined based on the total number of SNPs identified. Variants were further categorised by functional classes including missense, nonsense and silent mutations. Annotation of these variants was performed using SnpEff v5.0.e. The identified variants were then compared with gene and exon boundaries, protein coding regions, non-coding regions and untranslated regions (UTRs) to determine their genomic locations in Ankamali pigs. Functional annotation was conducted specifically within the coding regions of the Ankamali genome to establish their genomic positions, mutation types, alternate bases, altered codons and resulting amino acid changes.

RESULTS AND DISCUSSION

To gain insight into the genomic characterisation, we conducted whole-genome sequencing on 12 Ankamali pigs, marking the first comprehensive analysis of genome-wide variants in this breed. The whole genome sequencing of Ankamali pigs yielded 205.66 Gbs of raw sequence data as paired-end 150 bp sequences, generating approximately 1.37 billion reads (1,371,054,804 reads). About 95.47% of R1 reads and 94.27% of R2 reads were obtained using paired-end sequencing, with a base quality score (Q) greater than 30. Following quality control, which retained 1,360,094,278 reads, these were mapped to the Sus scrofa 11.1 reference genome sequences (Suss11.1, GenBank assembly GCF_000003025.6), with 99.77 per cent

(1,357,023,508 reads) aligning accurately to the reference genome assembly. Post-alignment, the reads were sorted and any duplicate reads were identified and removed from the alignment dataset. In this analysis, a total of approximately 26.6 million variants were identified. These variants included 21,303,641 (80.7 per cent) single nucleotide polymorphisms (SNPs), 3,056,981 (11.4 per cent) insertions and 2,243,967 (8.4 per cent) deletions, among others. The distribution of variant types is detailed in Table 1. Michelle *et al*. (2023) reported that, n Ankamali pigs, the total genome length obtained exceeded 2.5 billion bases, with an average of one genetic variant detected for every 94 bases sequenced. Within the Ankamali pig genome, 69.58 per cent of identified SNPs were transitions, while 30.41 per cent were transversions. The observed Ts/Tv ratio for SNPs was 2.29, indicative of accuracy consistent with recommendations for whole-genome sequencing SNP analysis (DePristo *et al*., 2011). Similar studies in cattle have been referenced (Stothard *et al*., 2011; Choi *et al*., 2014; Choi *et al*., 2015).

Annotation of 26,604,589 single nucleotide variants (SNVs) in the genome of Ankamali pigs revealed that 11,966,610 variants (45.09 per cent) were located in intronic regions, with 10,056,432 variants (37.79 per cent) found in intergenic regions. The remaining variants included 2,139,505 (8.04 per cent) upstream gene variants, 875,490 (3.29 per cent) downstream gene variants, 231,530 (0.87 per cent) 3-prime UTR variants, 101,748 (0.38 per cent) 5-prime UTR variants and 73,475 (0.28 per cent) intragenic variants. This genomic region distribution in Ankamali pigs is documented in Table 2. Only a small portion of the genome, approximately two percent, codes for proteins. These findings are consistent with several whole-genome sequencing studies demonstrating that more than half of SNVs in cattle and pigs are located in non- coding regions (Mei *et al*., 2018; Zhang *et al*., 2020). Bartonicek *et al*. (2017) noted that non-coding regions such as intergenic and intron regions play critical roles in various cellular processes, including gene expression, transcriptional control and gene splicing.

The identified variations were annotated to evaluate their impact on corresponding amino acid codons. This annotation process identified specific altered codons and aminoacids resulting from each variation, categorising mutations into types such as silent, missense and nonsense mutations with their respective counts. Table 3 presents the classification of variant numbers by functional class in the Ankamali pig genome. Among all SNPs located in exon regions, 66.19 per cent were classified as silent mutations, 33.52 per cent as missense mutations and

0.29 per cent as nonsense mutations. The missense to silent mutation ratio of 0.50, comparable to findings in Nero Siciliano pigs (D'Alessandro *et al*., 2019), serves as a metric for evolutionary rates according to Hu and Banzhaf (2008). This ratio indicates purifying selection in Ankamali pigs, suggesting the removal of harmful mutations and a slowed rate ofamino acid changes over evolutionary time.

Variants can significantly impact protein structure, with the nature and scope ofvariation playing crucial roles. Over 150 million variant effects were identified and classified into three categories: impact, region and type. According to the impact classification (Table 4), high-impact variants were relatively rare (0.01 per cent), while low-impact (0.26 per cent) and moderate-impact (0.10 per cent) variants were more prevalent in Ankamali pigs. Over 99% of these effects were categorised as modifiers. In terms of type classification (Table 5) within the Ankamali pig genome, nine high-impact variants, seven modifier impact variants, six moderate-impact variants and five low-impact variants were identified. High-impact variants can significantly disrupt protein function, potentially causing truncation or loss of function, whereas low-impact variants are assumed to have minimal effect on protein behaviour. Modifier impacts affect non-coding genes, where prediction is challenging or unsupported by evidence. Based on the region classification (Table 6) in the Ankamali pig genome, over 40% of effects were found in intronic and transcript regions. These classifications provide insights into how variants influence different aspects of genetic function and protein structure in Ankamali pigs.

CONCLUSION

The current study has generated the complete genome sequence of Ankamali pigs, a native variety from Kerala. The study highlights a higher genomic variability in Ankamali pigs compared to the reference genome, underscoring their distinct phenotypic and genetic characteristics. The study has identified potential variants that could be pivotal for future research on the advantageous traits of Ankamali pigs. These findings are crucial for developing enhanced conservation strategies aimed at preserving and utilising the unique genetic resources of this variety effectively.

ACKNOWLEDGMENTS

The authors acknowledge Kerala Veterinary and Animal Sciences University (KVASU) and All India Coordinated Research Project (AICRP) on Pig for providing the facilities for the conduct of research. We thank Clever gene Labs Pvt Ltd, Bengaluru, Karnataka for the sequencing and bioinformatics services

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RESEARCH ARTICLE
JIVA 22 (2) August 2024
https://doi.org/10.55296/JIVA/22.2.2024.42-49

Received on 29/06/2024
Accepted on 09/08/2024

OCCURRENCE OF MIXED INFECTIONS OF *CAPILLARIA* SPP. AND *HETERAKIS* SPP. IN TURKEYS AND ITS CONTROL

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ABSTRACT

Native turkeys kept as domestic bird in rural environments can be infected with bacterial, viral and parasitic agents. Parasites affect health and production of turkeys and cause economic loss. *Capillaria* spp. and *Heterakis* spp. are the major helminths reported to be highly pathogenic for turkeys in both deep-litter systems and free-range systems. The most important role of*H. gallinarum* is its capability of transferring the protozoan *Histomonasmeleagridis*, responsible for infectious enterohepatitis in fowls. Hence the characterisation of species is necessary to adopt proper management and control measures.The present report documents the morphological identification of nematodes collected during postmortem examination of turkeys in a small holder farm at Thrissur.A markedly dilated caecum with severe catarrhal exudates was observed.The examination of intestinal contents revealed parasitic ova along with adult worms. The recovered adult worms were identified as *Baruscapillaria obsignata* and *Heterakis gallinarum*. Faecal sample examination of live birds in the farm also revealed mixed infections. The birds with infection were advised treatment with pyrantel pamoate at a dose rate of 7 mg/kgper orally once.

Keywords: Turkey, *Heterakis, Capillaria,* Nematode

INTRODUCTION

Poultry industry represents the pinnacle of vertical integration and intensification among animal production industries. In numerous countries around the globe, poultry has emerged as a dominant component of the livestock industry (Udoh *et al*., 2014). The sector encompasses a plethora of domesticated bird species, including chickens, ducks, geese, guinea fowl, and turkeys. Turkey is a large poultry bird, and its popularity among peasant farmers has been increasing in recent times due to its high rate of production, its high

feed conversion rate, and the relatively low land requirements necessary for its rearing. The gastrointestinal tract (GIT) of the turkey is commonly invaded by a range of parasites, including protozoa, nematodes,cestodes and trematodes among which helminth infections are most common (Soulsby, 1982). The presence of parasites in the GIT of turkeys may result in a number of undesirable outcomes including loss of appetite, emaciation, diarrhoea, anaemia, reduced egg production, and retarded growth, all of which have the potential to reduce the economic value of the animals.

Capillariosis is a helminthic infection caused by a large group of trichurid nematodes, comprising approximately 300 species that are distributed globally and parasitise all vertebrates (Barathidasan *et al*., 2014). The avian capillarid worms belong to different genera, including *Baruscapillaria*, *Capillaria*, *Echinocoleus*, *Eucoleus*, *Ornithocapillaria*, *Pterothominx* and *Tridentocapillaria* (Palanivelu *et al*., 2016). These tiny nematodes, commonly referred to as hair worms, are classified within the super family Trichinelloidea, family Capillaridae, and subfamily Capillarinae (Barathidasan *et al*., 2014; Palanivelu *et al*., 2016). These parasites are known to infest various segments of the GIT of domestic poultry, causing severe enteric disease and associated mortality. Helminth infections caused by worms of the genus *Heterakis*, primarily inhabit the interior of the caeca of chickens, turkeys, guinea fowls, quails, ducks, pheasants, and geese. Three species are considered particularly prevalent in poultry *viz*., *Heterakis gallinarum*, *H. dispar*, and *H. isolonche* (Park and Shin, 2010). The most significant role of *H. gallinarum* is its ability to transmit the protozoan *Histomonas meleagridis* that is highly fatal in turkeys.

In this study we describe concurrent infection of *Capillaria* spp. and *Heterakis* spp. in turkeys along with successful control of mixed infection in a small holder turkey farm.

MATERIALS AND METHODS

The present study was conducted in a small holder turkey farm in Thrissur with a history of sudden death of birds. At necropsy, intestine and caecum were collected. Intestinal contents were examined by direct and conventional sedimentation methods. The adult parasites werecollected, washed with normal saline and then cleared withlactophenol.Morphological examination and identification involved morphometry of the eggs as well as dimensional study of the adult parasites (Palanivelu *et al*., 2016: Park and Shin, 2010). The length and width of the eggs and adult worms weremeasured using

10 X objective of microscope (Magnus, MX21iLEDFS121) and depicted in millimetres (mm).

The faecal samples of live birds (n=5) were collected and transported to laboratory for further examination. Sedimentation was carried as per the protocol described by Soulsby (1982) to identify parasite infections.

RESULTS AND DISCUSSION

Markedly dilated intestinal tract stuffed completely with adult parasites and catarrhal exudate was observed at necropsyas reported by Park and Shin (2010). It could be reasonably concluded that the damage caused by these worms to the mucosal layer of the digestive tract impairs digestion and absorption of nutrients, which subsequently resulted in a reduction of appetite and an increased incidence of diarrhoea. In severe cases, this leads to debility, and ultimately death of the affected bird (Palanivelu *et al*., 2016).

Mucosal scrapings and contents when observed under microscope revealed thepresence of adult worms and barrelshaped eggs with prominent bipolar plugs consistent with morphology of *Capillaria* spp. (Figure 1). Ten adult helminths were recovered that resembled nematodes and were further processed.

Eight worms that were thin and hair like revealed a stichosome glandular oesophagus consisting of variable number of stichocytes at their anterior part (Figure 2). The oesophagus of male worms (n=2) were more than half of the whole length of the parasite, while that of females (n=6) were shorter. Theposterior end of male had pseudobursa with a single, long non-spiny spicular sheath and two ventrolaterallobes without caudal alae (Figure 3). The female wormsshowed vulva region at middle of the body and theeggs were having prominent bipolar plugs (Figure 4). Based on these morphological features and location oflesions/predilection site in the host, the parasite was identified as *Baruscapillaria obsignata* as previously documented by Palanivelu *et al*. (2016) in India. Micro morphometric details of egg and adult worms are given in Table 1.*Baruscapillaria obsignata* can be differentiated from other capillarids affecting turkeys based on morphometry (Taylor *et al*., 2016). Enteric capillariosis caused by *B. obsignata* is known to occur in all birds belonging to Anseriformes, Ciconiiformes, Columbiformes, Galliformes, Piciformes and Psittaciformes (Jortner *et al*., 1967; Rickard and Pohl, 1969;Pinto *et al*., 2004; Pinto *et al*., 2008; Yabsley 2008; Park and Shin, 2010; D'Avila *et al*., 2011). In India, Singh *et al*. (2006) and Das *et al*. (2015) have detected capillarid eggs in faeces of

few turkey birds. *Capillaria* spp. infections can be highly pathogenic to birds in deep litter or free-range systems where large numbers of infective eggs can accumulate in litter or soil. (Park and Shin, 2010). These worms are reported to cause lower growth rates, decreased production and reduced fertility in birds (Rickard and Pohl, 1969). The intestinal thickening hasbeen attributed to chronic and repeated infection, high worm load and frequent diarrhoea (Moravec *et al*., 1987). The present study is the first report from Kerala that describes the morphology and morphometry of *B. obsignata* in turkeys.

Table 1. Micro morphometry of egg and adult worms of *Capillaria* spp.

Micrometry parameters	Measurements (mm)	
	Male	Female
Body length	10	15
Body width	0.05	0.042
Spicule length	1.11	
Spicule width	0.02	
Egg length	0.052	
Egg width	0.031	

Two adult female worms medium to large and creamy white were retrieved from caecum along with thick, smooth shelled, ellipsoidal ova. Mouth opening was bordered by tri-radiate lips. The dorsal lip was slightly wider than the sub-ventral lips. Cuticle was distinctly striated. Lateral alae extended along the whole body. The oesophagus was cylindrical and slightly extended toward posterior end with a posterior bulb (Figure 5). Morphometric examination revealed the following characters: Body 10mm long and 0.37mm wide. Oesophagus 0.8 mm long. Nerve ring located at 0.07 mm from the anterior end. Vulva was situated in the middle third of the body at 3.8 mm from the anterior extremity of the body, formed as a transverse slit with indistinct borders. The vagina was short and branched into two diverging uterine branches filled with embryonated eggs. Tail was slender and 0.88 mm long (Figure 6). Based on these morphological and morphometric features as well as location of lesions/predilection site in the host, the parasite was identified as *H. Gallinarum* (Suresh, 2003).The analysed specimens in this study exhibited the general characteristics typically associated with the genus *Heterakis*. With the exception of minor variations in the measurements, the morphological features of the detected nematode species in the current study were found to be in close agreement with those described in the earlier publications by Park and Shin (2010) and Abdel-Gaber *et al*. (2023) on *H. gallinarum*. The discrepancy in measurements may be attributed to the birds from which the parasites were collected and the methods of preparation for examination, which is consistent with the opinion of Al-Moussawi (2016). The helminth is associated with transmission of

Fig. 1. Barrel shaped eggs with bipolar plugs

Fig. 2. Oesophagus consisting of variable number of stichocytes of *B. obsignata*

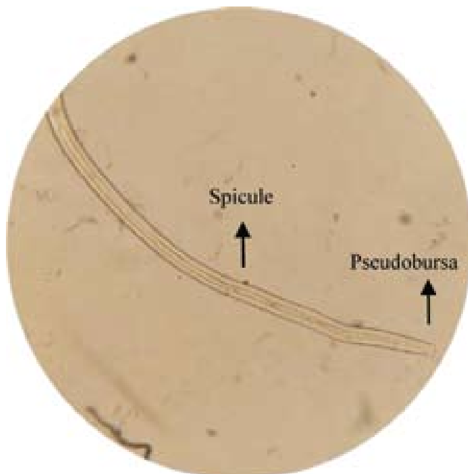

Fig. 3. Posterior end of *B. obsignata* male with single spicular sheath and pseudobursa with two ventrolateral projections

Fig. 4. Female *B. obsignata* showing vulva region and barrel shaped eggs with bipolar plugs in uterus

Fig. 5. Oesophagus with posterior bulb and valvular apparatus of female *H. gallinarum*

Fig. 6. Female *H. gallinarum* tail end

Histomonas meleagridis, a fatal protozoan in turkeys (Park and Shin, 2010). Hence this infection needs to be seriously viewed.

Common anthelmintic drugs like fenbendazole, febantel andlevamisole are considered highly efficacious in avian species (Yabsley, 2008). In this study, faecal sample of live birds from the same flock was examined further which revealed mixed infection. Infected birds were treated with pyrantel pamoate at a dose rate of 7 mg/kg per orally once, which controlled the mortality. Subsequent examination of faecal samples of treated birds after two weeks revealed no parasitic ova.

The morphology and ecology of the parasite has traditionally formed the basis for the taxonomy of nematode parasites (Bobrek *et al*., 2019). However, recent advances in molecular techniques have enabled the development of novel parasitological diagnostic tools, which are more sensitive and specific than conventional diagnostic methods (Tarbiat *et al*., 2021). Further characterisation of species and continued monitoring in farms are important in attaining sustained control of helminth.

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