Title Page

Authors:

Amna Arshad Bajwa[1], Jordan P. Cuff[2], Muhammad Imran[1], Saher Islam[1,] Riffat Mansha[1], Kamran Ashraf[3], Arman Khan[1], Muhammad Imran Rashid[3], Muhammad Yasir Zahoor[1], Waseem Ahmad Khan[4], Habib Ur Rehman[5], Asif Nadeem[1], Pablo Orozco-terWengel[2,] *****, Wasim Shehzad[1]*****

Title:

Assessment of nematodes in Punjab Urial (*Ovis vignei punjabiensis*) Population in Kalabagh Game Reserve: Development of a DNA barcode approach

Authors Affiliation:

1: Institute of Biochemistry & Biotechnology, University of Veterinary & Animal Sciences, Lahore 54000, Pakistan

2: School of Biosciences, Cardiff University, Cardiff CF10 3AX, Wales United Kingdom.

3: Department of Parasitology, University of Veterinary & Animal Sciences, Lahore 54000, Pakistan

4: Department of Wildlife & Ecology, University of Veterinary & Animal Sciences, Lahore 4942, Pakistan

5: Department of Physiology, University of Veterinary & Animal Sciences, Lahore 54000, Pakistan

Corresponding Authors:

wasim.shehzad@uvas.edu.pk

Ph: 042-99213510
Fax: 042-99213303

orozco-terwengelpa@cardiff.ac.uk
Ph: +442920875026

Abstract

Punjab Urial (*Ovis vignei punjabiensis*) is a wild sheep of Pakistan, considered a vulnerable species by IUCN. Major threats to Urial populations include habitat loss and poaching, causing severe declines in its population. Nematode infections may also compromise Urial survival, but little is known about Punjab Urial gastrointestinal nematodes. In this study, a novel DNA barcoding approach was developed using *ITS-I* as a target region, with a primer pair designed to amplify frequently reported nematode species for small ruminants. The novel primer pair was validated *in silico* and *in vitro* and subsequently used to determine the presence of nematodes in Punjab Urial samples from Kala Bagh Game Reserve, District Mianwali (Pakistan). DNA barcoding revealed a higher prevalence of *Haemonchus contortus* (73.91%), *Trichuris ovis* (16.30%) and *Trichostrongylus axei* (3.26%) in Punjab Urial. This study demonstrates that the novel DNA barcoding approach is a robust tool to detect nematode parasites from faecal samples of Punjab Urial. This method can be used to detect nematode infections in wild and domestic hosts for surveillance and population conservation.

Keywords:

Nematode, Barcoding, Internal transcribed spacer, Diagnosis, DNA

Introduction

Wild ungulates are a key component of the ecosystems they inhabit, but in many regions of the world they are threatened by poaching, habitat fragmentation and competition with domestic livestock (Ito et al. 2013). Conservation of Asiatic ungulates is often hampered by poor knowledge of basic population dynamics and quantification of specific threats. Punjab Urial (*Ovis vignei punjabiensis*) is a species of special interest among wild mammals of Pakistan. This wild sheep is endemic to Northern Punjab (Pakistan) and is listed as vulnerable by the IUCN Red List of Threatened Species (IUCN, 2018).

Urial populations have declined by 30% over the last three generations (Valdez 2008) and are struggling against severe hunting and poaching pressures (Awan et al. 2006; Khan et al. 2015). Whilst competition among sympatric and anthropogenic effects threaten these declining populations, disease provides an even greater threat (Dhollander et al. 2016; Gortazar et al. 2015; McCallum and Dobson 1995; Walker and Morgan 2014). Diseases, even of mild severity, have a more profound influence on wild sheep population dynamics than any other factor (Gross et al. 2000). Gastrointestinal nematode (GIN) infection is one of the major prevailing diseases among wild ungulates resulting in high morbidity and mortality (Albon et al. 2002; Charlier et al. 2009; Hudson 1986). Multiple nematode species may infect their hosts simultaneously, both in wild and domestic ruminants (Sim et al. 2010). Very few studies have focused on the diseases affecting Urial populations (Shahzad et al. 2015), with the first reports dating from work carried out between 2001-2002 (Awan et al. 2005). Studies on parasitic infections such as GIN are frequently based upon observation without genetic validations of the species identified and to date no study has yet reported nematodal infection in Urial sheep using molecular methods.

For wild mammals there is a need to design general approaches to GIN detection able to simultaneously detect different nematode species. In the present study, barcoding primers were designed to investigate the presence of GIN in wild Punjab Urial. The new primers were tested on a variety of nematodes known to infect ovines and where validated on the three most important GIN for Punjab Urial, i.e. *Trichostrongylus axei, Haemonchus contortus* and *Trichuris ovis*. These new primers will facilitate the use of non-invasive procedures for monitoring nematode infections in wild populations of Punjab Urial and possibly other species. The method described here will dramatically reduce the labor and inaccuracy associated with current methodologies. With improved monitoring strategies in place, mitigating the pressures on these declining populations is far easier, thus enhancing their chance of survival.

Methodology

A total of 114 putative Urial sheep faecal samples were collected between April 2016 and May 2017 from Kala Bagh Game Reserve, District Mianwali (Pakistan). All DNA extractions were performed using the GeneAll Exgene Stool SV Kit following the manufacturer's instructions. A Urial-specific primer pair (Urial_F / Urial_R), amplifying a 100-bp fragment (including primers length) of the mitochondrial *cytochrome-b* gene (Table: I) was used to distinguish Urial faeces from other ungulates co-occurring in the study area, e.g. domestic sheep (*Ovis aries*) and goats (*Capra hircus*). The samples successfully amplified with the Urial-specific primer pair (F/R) were selected for further analyses.

A database of nematode *ITS-I* sequences was compiled for use with ecoPCR (Ficetola et al. 2010). All *Haemonchus, Trichuris, Trichostrongylus, Ostertagia, Teladorsagia, Cooperia, Nematodirus, Marshallagia* and *Oesophagostomum ITS-I* sequences available on GenBank

(NCBI) were downloaded on 20th March 2018 and only those sequences over 200 bp long were retained. Where multiple sequences were present per species, only one was included in the database to ensure even representation across taxa. The sequences were aligned in Geneious to ensure that they fully covered both primer sites and those that did not were removed. The final database contained *ITS-I* sequences for 84 nematode species, including the target species *Trichostrongylus axei*, *Haemonchus contortus* and *Trichuris ovis*. Six simulations were run with the database via ecoPCR allowing three or five mismatches and with zero, two or three precise matches required at those bases closest to the 3' end of both primers. The percent coverage by the primer pairs of the database was calculated for each simulation.

A fragment of approximately 140bp was amplified using the designed primer set (Nem-BRCD_F/ Nem-BRCD_R; Table: I). Positive and negative controls were also used in all reactions to monitor validation and any contamination, and all reactions were carried out in five replicates. The PCR products were sequenced using an ABI- 3130 Genetic Analyzer. The sequences obtained via Sanger Sequencing (ABI-3130xl DNA Analyzer) have been submitted to GenBank (Accession No: MG243367-MG243370).

Results and Discussion

The conservation of wildlife in a given area requires comprehensive information about the present anthropogenic and non-anthropogenic threats, such as disease. This study aimed to investigate nematode infection of Urial populations via non-invasive genetic sampling and novel barcoding primers. Of the 114 putative faecal samples collected from Kala Bagh Game Reserve, 92 samples were identified as Punjab Urial using Urial-specific primers (Urial_F / Urial_R). Punjab Urial samples were further analyzed with the novel barcoding primers and showed a predominant occurrence of *Haemonchus* infection in the Urial sheep. Overall, the percentage of detection was significantly higher (~73%) for *H. contortus* than in previous studies conducted on domestic and wild sheep (Red Maasai; (Gatongi et al. 1998; Pandey et al. 1994) (Asif et al. 2008; Zapata Salas et al. 2016). Prevalence of *T. axei* was notably lower than previously reported (Raza et al. 2014; Tan et al. 2014). The reported prevalence does not, however, directly relate to previous studies as the host animal and geographic conditions vary significantly (Bibi et al. 2017; Önder et al. 2016; Qasim et al. 2016; Raza et al. 2014; Rizwan et al. 2017). We could not amplify six samples using PCR, possibly due to a lack of GINs, failure during DNA extraction, the presence of only degraded DNA, or the species present not being amplified by these primers.

The results of the *in silico* ecoPCR analyses varied with the conservativeness of the simulation, with percent coverage of the nematode database varying between 19.05% and 34.52% (Table: II). In all simulations, *Trichostrongylus. axei* and *H contortus* were successfully amplified, but *Trichuris ovis* was not (Table: I; Supplementary material). However, the primers successfully amplified all three target species during *in vitro* testing. The percentage coverage of the database described here with the *in silico* analysis suggests that these primers are useful to identify a wide array of nematode species in addition to those for which they were initially designed. Although

the results of such *in silico* tests are often conservative, they can be interpreted as a guide or a lower threshold for the amplification potential of a primer pair, as evidenced by the lack of success in amplifying *T. ovis in silico,* but a successful amplification *in vitro*. The results indicate that mismatches within two bases of the 3' end limit the generality of the primers, specifically the first base at the 3' end of the forward primer. If using the primer pair for applications considering more nematode species, substituting the "T" base for a degenerate "Y" (representing both "C" and "T") would increase the percentage database coverage by approximately 5% in each simulation enabling amplification of approximately 24-39% of the species. However, such modification of the primers would become valuable if these are used for metabarcoding via next-generation sequencing. Furthermore, if the primers amplify the target species at approximately equal rates as determined by the complementarity of the primer sequence to the template DNA, the metabarcoding approach could be used in a quantitative manner enabling estimating the amount of the parasite in a DNA sample (Pinol et al. 2018) thereby providing quickly more accurate results than classic morphological techniques.

This study showed that DNA barcoding using these novel primers can achieve species-level identification, and it is likely to facilitate identification of previously unknown and unreported GIN species in ovines. Genetically uncharacterized species can be problematic for barcoding due to their sequences being absent from public DNA databases. Fifteen samples in this study were identified as local *Trichuris ovis* variants when compared with the sequences obtained from NCBI and had 100% similarity with the positive control sample obtained from the slaughter house in Lahore. However, for other species the closest match in a database may only be possible at the genus or family levels, thus highlighting the need to prepare and curate local parasite genetic DNA databases to enable overcoming these problems. Due to the use of Sanger

sequencing in this study, only the most abundant nematode species was identified from each faecal sample, thus no mixed infections were identified despite the primers being able to amplify different species of nematodes. The possible incidences of mixed infections mean that the prevalence values are likely to underestimate the number of Urial infected with each nematode. Furthermore, while the primers described here could be modified to quantify nematode infection by a particular species using real-time PCR (Bell and Ranford-Cartwright 2002; von Samson-Himmelstjerna et al. 2002), we argue that the use of these primers in combination with next generation sequencing, as done in metabarcoding studies, is a better approach to capture nematode diversity and simultaneously effectively monitor both mixed and single infections in Punjab Urial and other wild and domestic ungulates sharing similar nematode infections.

Our results reported that *H. contortus* is highly prevalent (~73%) in Punjab Urial population while *Trichuris ovis* and *T. axei* (~16% and ~3% respectively) showed lower prevalence. This study presents a novel and non-invasive approach to monitor nematode prevalence in the Urial population in Kala Bagh Game Reserve, Mianwali.

References

Albon S, Stien A, Irvine R, Langvatn R, Ropstad E, Halvorsen O (2002) The role of parasites in the dynamics of a reindeer population Proceedings of the Royal Society of London B: Biological Sciences 269:1625-1632

Asif M, Azeem S, Asif S, Nazir S (2008) Prevalence of gastrointestinal parasites of sheep and goats in and around Rawalpindi and Islamabad, Pakistan J Vet Anim Sci 1:14-17

Awan G, Ahmad T, Festa-Bianchet M (2005) Disease spectrum and mortality of Punjab urial (Ovis vignei punjabiensis) in Kalabagh Game Reserve Pakistan Journal of Zoology 37:175

Awan GA, Festa-Bianchet M, Ahmad T (2006) Poaching, recruitment and conservation of Punjab urial Ovis vignei punjabiensis Wildlife Biology 12:443-449

Bell AS, Ranford-Cartwright LC (2002) Real-time quantitative PCR in parasitology TRENDS in Parasitology 18:338-342

Bibi R et al. (2017) Phenotyping and Prevalence of Haemonchus contortus (Nematoda: Trichostongylidae) in Ruminants from Endemic Areas of Pakistan: Influence of Host Species and Geographical Area on Phenotypic Traits of Worms Pakistan Veterinary Journal 37

Charlier J, Höglund J, von Samson-Himmelstjerna G, Dorny P, Vercruysse J (2009) Gastrointestinal nematode infections in adult dairy cattle: impact on production, diagnosis and control Veterinary Parasitology 164:70-79

Dhollander S et al. (2016) Assessing the potential spread and maintenance of foot-and-mouth disease virus infection in wild ungulates: general principles and application to a specific scenario in Thrace Transboundary and emerging diseases 63:165-174

Ficetola GF et al. (2010) An In silico approach for the evaluation of DNA barcodes BMC Genomics 11:434 doi:10.1186/1471-2164-11-434

Gatongi P, Prichard R, Ranjan S, Gathuma J, Munyua W, Cheruiyot H, Scott M (1998) Hypobiosis of Haemonchus contortus in natural infections of sheep and goats in a semi-arid area of Kenya Veterinary parasitology 77:49-61

Gortazar C, Diez-Delgado I, Barasona JA, Vicente J, De La Fuente J, Boadella M (2015) The Wild Side of Disease Control at the Wildlife-Livestock-Human Interface: A Review Frontiers in Veterinary Science 1 doi:10.3389/fvets.2014.00027

Gross JE, Singer FJ, Moses ME (2000) Effects of disease, dispersal and area on bighorn sheep restoration Restor Ecol 8:25-37

Hudson PJ (1986) The effect of a parasitic nematode on the breeding production of red grouse The Journal of Animal Ecology:85-92

Ito TY, Lhagvasuren B, Tsunekawa A, Shinoda M, Takatsuki S, Buuveibaatar B, Chimeddorj B (2013) Fragmentation of the Habitat of Wild Ungulates by Anthropogenic Barriers in Mongolia PLOS ONE 8:e56995 doi:10.1371/journal.pone.0056995

Khan W, Ahmed M, Yaqub A, Ali H, Arshad M (2015) Distribution and population status of Punjab Usrial, *Ovis vignei punjabiensis* (mammalia: Bovidae), in soan valley, salt range, Punjab, Pakistan Journal of Animal and Plant Sciences 25:851-859

McCallum H, Dobson A (1995) Detecting disease and parasite threats to endangered species and ecosystems Trends in Ecology & Evolution 10:190-194 doi:https://doi.org/10.1016/S0169-5347(00)89050-3

Önder Z, Yildirim A, Incİ A, Düzlü Ö, Çİloğlu A (2016) Molecular Prevalence, Phylogenetic Characterization and Benzimidazole Resistance of Haemonchus contortus from Sheep Kafkas Univ Vet Fak Derg 22:93-99

Pandey V, Ndao M, Kumar V (1994) Seasonal prevalence of gastrointestinal nematodes in communal land goats from the highveld of Zimbabwe Veterinary Parasitology 51:241-248

Pinol J, Senar MA, Symondson WOC (2018) The choice of universal primers and the characteristics of the species mixture determine when DNA metabarcoding can be quantitative Mol Ecol doi:10.1111/mec.14776

Qasim HM, Avais M, Durrani AZ, Khan MA, Shahzad AH (2016) Dynamic Dispersal of Haemonchosis, its Treatment and Effect on Blood Profile of Small Ruminants of Lodhran District, Punjab, Pakistan Pakistan Journal of Zoology 48:755-761

Raza MA, Younas M, Schlecht E (2014) Prevalence of gastrointestinal helminths in pastoral sheep and goat flocks in the cholistan desert of pakistan The Journal of Animal and Plant Sciences 24:127-134

Rizwan HM, Sajid MS, Iqbal Z, Saqib M (2017) Point prevalence of gastrointestinal parasites of domestic sheep (*Ovis aries*) in district sialkot, punjab, pakistan The Journal of Animal and Plant Sciences 27:803-808

Shahzad S et al. (2015) Comparative hemato-biochemical study on theileriosis in naturally infected punjab urial (Ovis vignei punjabiensis) and domestic sheep (Ovis aries) in Pakistan Journal of animal and plant sciences 25:472-476

Sim KA, Hoar B, Kutz SJ, Chilton NB (2010) Amplification of the second internal transcribed spacer ribosomal DNA of individual trichostrongylid nematode larvae by nested polymerase chain reaction Journal of veterinary diagnostic investigation 22:433-437

Tan TK, Panchadcharam C, Low VL, Lee SC, Ngui R, Sharma RS, Lim YA (2014) Co-infection of Haemonchus contortus and Trichostrongylus spp. among livestock in Malaysia as revealed by amplification and sequencing of the internal transcribed spacer II DNA region BMC veterinary research 10:38

Valdez R (2008) Ovis orientalis The IUCN Red List of Threatened Species

von Samson-Himmelstjerna G, Harder A, Schnieder T (2002) Quantitative analysis of ITS2 sequences in trichostrongyle parasites International Journal for Parasitology 32:1529-1535

Walker JG, Morgan ER (2014) Generalists at the interface: Nematode transmission between wild and domestic ungulates International Journal for Parasitology: Parasites and Wildlife 3:242-250 doi:https://doi.org/10.1016/j.ijppaw.2014.08.001

Zapata Salas R, Velásquez Vélez R, Herrera Ospina LV, Ríos Osorio L, Polanco Echeverry DN (2016) Prevalence of gastrointestinal nematodes in sheep and goat production systems under confinement, semi-confinement and grazing in municipalities of Antioquia, Colombia Revista de Investigaciones Veterinarias del Perú (RIVEP) 27:344-354 doi:10.15381/rivep.v27i2.11647