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A molecular epidemiological investigation of *Ascaris* on Unguja, Zanzibar using isoenyzme analysis, DNA barcoding and microsatellite DNA profiling

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ABSTRACT

Ascariasis is of public health importance on the islands of Zanzibar (Unguja and Pemba). To shed light on the molecular epidemiology of this parasite, 68 *Ascaris* worms, obtained from 14 individuals in four Ungujan villages, were examined by isoenzyme analysis (ISA), DNA barcoding and microsatellite DNA profiling. ISA revealed genetic variation, which was confirmed by DNA barcoding. Nineteen worms recovered from individuals in Uganda were included for comparison. Sixteen unique DNA barcodes were identified, 15 on Unguja and three in Uganda with two shared between. These two barcodes were found in all four Ungujan villages. Worms from Tumbatu-Jongowe, an isolated village on an islet off Unguja, seemed particularly diverse. Within our barcodes, three exact matches were found with Chinese *Ascaris* retrieved from pigs, which is perhaps surprising given the present rarity of these animals on Unguja. Microsatellite profiling and population genetic analysis revealed further genetic diversity within our samples although population sub-structuring within Unguja was minor in comparison to that between Unguja and Uganda. As African *Ascaris* has not been subjected to detailed molecular scrutiny, this new diversity represents an important piece in its evolutionary jigsaw and such population markers are informative in monitoring worm dynamics during ongoing control.

1. Introduction

Ascariasis is a neglected tropical disease caused by infection with soil-transmitted giant roundworms of the genus *Ascaris*, typically designated *Ascaris lumbricoides*. Around 1.2 billion individuals are infected worldwide, with the majority of infections occurring in the developing world, particularly in Asia and sub-Saharan Africa.[1] Cases of ascariasis can, however, still be found in developed countries, including the United Kingdom, Canada, the USA and Denmark.[2–4] It is commonly held that pathology associated with ascariasis depends on the worm burden in the gut of the infected individuals; light infections, where few worms are present, are often asymptomatic. However, medium-heavy infections can often cause chronic

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diarrhoea, abdominal pain and nausea. There is strong evidence for an association between ascariasis and malnutrition and stunted growth in children.[5] Perhaps of most consequence are heavy worm burdens where a trapped bolus of worms may lead to intestinal obstruction, which can be fatal.[5]

Ascariasis is one of the targets of large-scale 'deworming' programmes, which are based on regular distribution of the anthelmintic drugs to school-aged children living in endemic areas.[6] The main aim of these programmes is morbidity control rather than control of infections *per se* and on the islands of Zanzibar, for example, there have been encouraging declines in disease prevalence in school children.[7] However, it is unclear how sustainable such programmes are in the long-term especially as there is ongoing concern that resistance may develop to the limited number of drugs which are available for mass chemotherapy. On Zanzibar there are concerns of poor parasitological performance of albendazole against *Ascaris* and *Trichuris*.[8] Developing a monitoring system to assess changes in parasite population structure is important to provide a more robust assessment of the performance of chemotherapeutic agents in the context of ongoing control. A more detailed understanding of the evolutionary origins and population genetics of parasites is needed as it can provide new insights into transmission dynamics and can be used to inform control programmes. Early studies on the genetic diversity of *Ascaris* were based on the analysis of isoenzyme variation,[9,10] which could identify genetic variation at several enzymatic loci. More recently, molecular techniques including analysis of restriction fragment length polymorphisms and microsatellites, along with DNA sequencing of mitochondrial and nuclear genes, have been used to study the genetic diversity of *Ascaris* from a variety of locations.[4,11–14]

In light of these molecular data, the distinction between two species of *Ascaris*, *A. lumbrioides* and *A. suum*, is often blurred.[15] Indeed, there is on-going debate as to whether they do actually represent two (sub-)species and whether *Ascaris* from pigs can infect humans and *vice versa*. Studies on *Ascaris* in Guatemala and China suggest that there is little cross-over and gene flow between human and pig *Ascaris*, despite the close proximity of humans and pigs in these settings.[13,14,16–18] Yet 4–7% of the worm population in sympatric areas may consist of hybrids[19] and molecular evidence indicates that ascariasis is a zoonosis in North America, Denmark and the United Kingdom with pigs acting as the main reservoir for human infection (Bendall, unpublished).[2,4] The possibility of cross-infection between pigs and humans is important not just in terms of transmission, but could also allow genetic traits, for example putative drug resistance alleles, to cross between porcine and human *Ascaris* populations.

To date there has been very little published on the population genetics of *Ascaris* in sub-Saharan Africa, the only data being on 18 worms from Madagascar.[2,16] Ascariasis continues to be a public health problem in many areas of sub-Saharan Africa, including in Zanzibar, where despite long term interventions[7,20], very young children are still at risk of infection. For example, Stothard et al.[21] established that there are 'hotspots' of infection across Unguja where local prevalence can be up to 60% in preschool children. During this survey a number of *Ascaris* worms were collected from individuals in four Ungujan villages[21] and the present study reports on isoenzyme, DNA barcoding and microsatellite DNA profiling analysis of these worms with the aim of gaining insights into the local genetic diversity of *Ascaris* on Unguja.

2. Materials and Methods

2.1. Ascaris worm collection

In the summer of 2006, an epidemiological survey for soil-transmitted helminths (STHs) was carried out in 10 villages on Unguja, representative of urban, semi-urban and rural environments.[21] In four of these villages (Kandwi, Ghana, Kizimbani and Tumbatu-Jongowe, see Figure 1A), the prevalence and intensity of *Ascaris* infection was sufficient to attempt *Ascaris* expulsion. A total of 68 *Ascaris* worms were collected from the stools of 14 individuals living in these villages who had been given pyrantel pamoate (Combatrin®, Pfizer, UK) at 10 mg/kg 24 hours previously.[21] To remove faecal material after expulsion worms were washed in water, blotted dry and the length and weight of each worm was determined as described.[21] For genetic comparisons, in July 2007 *Ascaris* worms were collected from infected individuals in Hamukaaka village, Kabale District, Uganda, a cool highland area characterised by high levels of human ascariasis and where the population had no history of deworming at the time of the study.[22] In total, 19 worms were retrieved from four individuals 24 hours after treatment with Combatrin® and were handled as described above.

2.2. Isoenzyme analysis

To attempt to relate the genetic diversity of *Ascaris* on Unguja to that previously reported in the literature, an isoenzyme analysis (ISA) was conducted using cellulose acetate electrophoresis (CAE) with Titan III® apparatus (Helena Laboratories, Gateshead, UK) on site in Unguja within the Helminth Control Laboratory Unguja (HCLU). ISA was performed on the day of worm collection using aqueous worm lysates from a 1 cm ground-up section of longitudinal muscle tissue from each worm. Briefly, longitudinal muscle was first excised and homogenised in TG buffer (0.025 M Tris-HCL, 0.192 M Glycine buffer [pH 8.0]), then centrifuged at 14 000 rpm for 10 minutes in a benchtop centrifuge. The supernatant was collected and stored at 4 °C before application onto a cellulose acetate plate.

Following Ibrahim et al.'s CAE analysis of *Ascaris* from Bangladesh,[9] visualisation of the following eight enzyme systems was attempted: glucose-6-phosphate dehydrogenase - **GDH** (EC 1.1.1.49), glucose-6-phosphate isomerase – **GPI** (EC 5.3.1.9), β-hydroxybutyrate dehydrogenase – **HBDH** (EC 1.1.1.30), isocitrate dehydrogenase - **IDH** (EC 1.1.1.42), malate dehydrogenase - **MDH** (EC 1.1.1.37), mannose-6-phosphate isomerase – **MPI** (EC 5.3.1.8), 6-phosphogluconate dehydrogenase – **6PGDH** (EC 1.1.1.44) and phophoglucomutase – **PGM** (EC 5.4.2.2.).