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Clinical aspects and control methods of parasite infections in ruminants.

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Desafíos para el control de los parásitos en los rumiantes

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Sin duda alguna, los parásitos son uno de los principales obstáculos en la producción de los rumiantes, comprometiendo tanto su bienestar como la seguridad alimentaria. La fase externa de los parásitos depende de las condiciones ambientales por lo que es previsible cambios asociados al calentamiento global, no sólo en la epidemiología y las consecuencias de los parásitos ya presentes en un determinado biotopo, sino que incluso es probable que se altere la biocenosis de muchos ecosistemas. El hombre también juega un papel en este sentido, asociado al comercio y transporte tanto de especies domésticas como silvestres que pueden introducir agentes y actuar como reservorios. Esto, en su conjunto, puede promover el desarrollo de zoonosis, algunas de ellas, parasitarias. La sociedad actual promueve también nuevos modelos como las granjas orgánicas/ecológicas en la que el uso de los químicos está restringido.

Esto, obviamente supone un mayor riesgo a las consecuencias de los parasitismos y exige la búsqueda de alternativas al control. Esto ya era prioritario, porque el desarrollo de resistencias a los antiparasitarios, en especial en los nematodos gastrointestinales, es dramático, estando descritas resistencias múltiples en todos los continentes. Existen distintas alternativas que incluyen un uso más sostenible de los fármacos, el control biológico y nutricional, y alternativas orientadas a estimular respuestas inmunitarias más eficaces, por ejemplo, mediante la vacunación y/o la selección de individuos resistentes.

Estas medidas probablemente se deberán aplicar de forma integrada, y adaptadas a los distintos escenarios. A modo de ejemplo, se discutirá la utilidad de la combinación de vacunas en razas ovinas canarias como alternativas para el control de estos parásitos.

Application of post genomic research to inform decisions in ruminant livestock veterinary practice.

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Around the world, livestock are kept to convert primary resources or industrial by-products to products that can be sold. The efficiency of livestock production is directly related to the efficiency of conversion of herbage or by-products mostly to meat or milk. The temperature and rainfall or irrigation conditions which favour herbage growth are the same as those which favour free-living stages of arthropod, protozoa and helminth parasites; while management aimed at optimising livestock production efficiency also favours contamination with and exposure to infective stages of these parasites. Consequently, parasitic infections, helminths in particular, are the foremost production limiting diseases of commercially farmed ruminant livestock.

The achievement and sustainability of efficient production requires planned animal health management, tailored to individual situations, in which the emphasis is proactive preventive management, as opposed to reaction to clinical disease outbreaks. The principles of helminth control are to reduce host exposure to infective stages to a level that will allow for the development of protective immunity, while enabling parasitic stage burdens to be manageable. This usually involves the integration of evasive host management and drug treatments; the timing of which is governed by the annual production cycle of the host, giving rise to the seasonal presence of naïve or less immunologically capable animals, and climatic conditions, favouring egg hatching, larval development and L3 survival. Sustainable roundworm control programmes for individual farmers are based on the common-sense application of knowledge of the farming system and inferences on the relationship between pasture contamination, the availability of infective larvae on pasture and the build-up of infection in animals.

Globally, many livestock keepers administer anthelmintic drugs with the unachievable and naïve single goal of eradicating parasites from their animals, preferring formulaic one-fits-all programmes for their use. However, these approaches don't work because of the parasites' different adaptations to changing climatic, human lifestyle, or livestock management effects on free-living stages, and the

exposure of parasitic stages to anthelmintic drugs. Over recent years, changes have become established in the epidemiology of *Teladorsagiosis*, *Haemonchosis* and *Nematodirosis* in small ruminants in northern Europe, while resistance to each of the single-active broad spectrum anthelmintics has now emerged and spread in multiple species wherever these drugs have been used. There is, therefore, a need for practical GI nematode control strategies integrating grazing management and anthelmintic drugs for use in specific agricultural contexts; incorporating anthelmintic resistance mitigation and exploring the exploitation of host genetic adaption, use of natural xenobiotics and vaccine development.

Genomic resources are needed to improve our understanding of these genetic adaptations as a basis for the iterative development of sustainable management advice. We have exploited the global importance, high fecundity and tractability of experimental infections of *Haemonchus contortus* as our model gastrointestinal nematode for genetic and post genomic approaches to identify loci conferring genetic adaptations. We now have a very high quality v4 283 Mb chromosomal level genome, which has 19,489 nuclear genes. A major problem for the assembly of overlapping contigs and scaffolds into a meaningful linear genome was the extremely high level of haplotypic diversity and polymorphism between individual nematodes. We overcame this by developing an innovative single parent genetic crossing method to produce near-isogenetic lines, one of which was used in our refined annotated genome and transcriptome. The complexity of the *H. contortus* genome, high fecundity and high levels of polymorphism mean that parasite adaptation in response to both favourable and unfavourable conditions has been inevitable.

Molecular tools are needed to study the emergence and spread of anthelmintic resistance. The investigation of possible associations between the resistance phenotype and polymorphisms in candidate genes was successful in identifying three SNP mutations in the isotype-1 β-tubulin gene, conferring benzimidazole resistance by preventing drug binding. However, studies of candidate genes have yet to unequivocally identify molecular loci responsible for resistance to other anthelmintic drug classes. This highlights the challenges of working with complex organisms, and that new approaches are needed to robustly identify genetic markers associated with resistance. The candidate gene approach depends either on prior assumptions about resistance mechanisms, which may differ from those of drug action; or on using genome-wide approaches to compare populations with different phenotypes, which is complicated by the high level of genetic polymorphism both within and between populations; or on the comparison of susceptible and experimentally selected resistant lines form a single isolate, which may not reflect mechanisms associated with field selection. Therefore, we have adopted alternative genetic crossing approaches using the *H. contortus* genome to search for genetic linkage to mutations that might confer heritable levamisole or macrocyclic lactone resistance.

Our first approach to identify resistance loci involved genetic crosses between populations the ivermectin susceptible genome project *H. contortus* strain and two independent ivermectin resistant strains. The progeny were exposed to ivermectin drug selection before four rounds of backcrossing to the susceptible strain parents. This resulted in the introgression of loci genetically linked to ivermectin resistance mutations into the susceptible parental genomic background. Genetic linkage was identified between a resistance locus and one microsatellite marker. We next undertook a genetic cross between populations of the drug susceptible genome isolate and another multi-drug resistant *H. contortus strain*. We developed a novel genetic linkage map based on whole genome sequencing of individual F1 progeny of a single female nematode. This demonstrated important aspects of the parasite's biology with reference to population genetics studies, such as polyandry and polyploidy. Extreme quantitative trait locus (x-QTL) analysis of anthelmintic drug selected progeny of this genetic cross showed loci linked to benzimidazole (chromosome II, containing the isotype-1 β-tubulin gene), leva-

misole (chromosome V, containing a candidate acetylcholine receptor gene) and ivermectin (an approximately 2 Mb locus on chromosome V, containing no candidate genes based on known or suspected mechanisms of macrocyclic lactone action). The high quality v4 *H. contortus* genome enabled the investigation of pairwise genetic diversity throughout ivermectin selected lines derived from the previous genetic backcrosses. This demonstrated the same single major genomic locus for ivermectin resistance on chromosome V in our backcrosses, based on South African- and Australian-derived resistant parental populations, and in our xQTL genetic cross, based on a north American-derived resistant parental population. Our attempts to refine the approximately 2 Mb locus for ivermectin resistance have been hindered by infrequent genetic cross over during meiosis and consequent low recombination rates. We have employed next-generation re-sequencing methods on individual parental and drug selected filial progeny of our genetic linkage mapping cross to identify haplotypic diversity and reduce the size of the genomic locus linked to the ivermectin resistance mutation. We have refined the selection of candidate genes for further investigation, using next generation transcriptomic methods such as RNA-Seq.

We have also undertaken dual strain crosses between genetically divergent *H. contortus* populations. Analyses of these has confirmed overlapping morphological differences between strains and reduced post-zygotic development and, or, viability in the hybrid progeny of paternal parents of the most genetically divergent strain. This is consistent with incipient speciation and is relevant to the study of population genetics resistance mutations in mixed-strain field populations of GI nematodes. This level of understanding of basic biology is important when considering the translation of population genetics research to the development of practical guidelines for sustainable gastrointestinal nematode control.

Our primary interest is in translating understanding, based on the above research of the selection pressures for emergence and spread of resistance mutations, to the field. We have used the isotype-1 β-tubulin SNPs conferring resistance in *H. contortus* and *Teladorsagia circumcincta* as proof of concept in this regard. We have evaluated population pyrosequencing and deep amplicon sequencing platforms to investigate the frequencies of isotype-1 β-tubulin SNPs, and have applied these to field populations of *H. contortus* and in Pakistani cattle and buffalo and of *H. contortus*, *Haemonchus* placei and *T. circumcincta* in Pakistani cattle and buffalo and Scottish sheep, respectively. Our results demonstrate different selective sweep patterns of emergence of benzimidazole resistance between H. contortus and H. placei in the Pakistani livestock, and subsequent spread with animal movements. We have explored a LAMP assay to detect frequencies of the presence, or absence, of the three isotype-1 β-tubulin SNPs in H. contortus populations; with encouraging preliminary results. This method could eventually be developed as a near pen-side test for use in under-resourced scenarios, albeit there are some significant challenges to overcome first.

To be meaningful, we need to consider the population genetics of resistance mutations in the field in the context of the gastrointestinal nematode species population structure that is present. The Illumina MiSeq-based deep amplicon sequencing clade V 'nemabiome' method developed by researchers at the Calgary Veterinary School has revolutionised our ability to study mixed species parasite populations. In our hands, the method is more sensitive, accurate and better suited to high throughput of multiple samples than conventional morphological speciation of coprocultured L3. We have used the 'nemabiome' method alongside faecal egg counts, isotype-1 β-tubulin SNP frequencies, microsatellite markers and qualitative data on livestock management, climate and gastrointestinal nematode control practices in our studies. Our results show high levels of genetic fixation of the isotype-1 β-tubulin mutations in T. circumcincta in Scottish sheep flocks, which will hinder future studies of environ-

mental, climatic, husbandry and drug treatment risk factors associated with their emergence. We are developing an amplicon sequencing method to explore mitochondrial DNA diversity in *H. contortus* and *T. circumcincta,* and a multiplex genome wide locus sequence typing method to explore genetic adaptations in *H. contortus.*

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Control inmunitario de las helmintosis de los rumiantes.

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Las helmintosis siguen siendo un determinante fundamental para la productividad de las explotaciones ganaderas de rumiantes en muchas zonas del mundo, que tienen que responder al reto de la creciente demanda de alimentos de origen animal, producidos de forma sostenible y segura. En ese marco, las limitaciones de los tratamientos antihelmínticos y los actuales planteamientos de salud ambiental están impulsando el desarrollo de nuevas estrategias de control, con una especial relevancia para la obtención de vacunas eficaces y comercialmente viables.

Se han realizado numerosos ensayos en muy diversos helmintos, habiéndose alcanzado resultados significativos en algunos nematodos gastrointestinales (vacuna comercial para *Haemonchus contortus*; protección consistente en vacunas multiantigénicas para *Teladorsagia circumcinta*). En otros parásitos de gran relevancia (*Fasciola hepatica*), los resultados no han sido tan determinantes. En conjunto, resulta claro que se han hecho avances muy notables y existe un amplio consenso en considerar una serie de puntos claves para continuar la progresión.

El primero de ellos es el conocimiento completo y detallado de la interacción parásito-hospedador en todas las fases de la infección, en el papel de las respuestas innatas como determinantes de las respuestas adquiridas y en los mecanismos inmunomoduladores que algunos parásitos inducen desde las instancias iniciales de la infección. Un aspecto crítico es la constatación de los mecanismos efectores de protección, ya sea por destrucción parasitaria, temprana o tardía, o por interferencia con los procesos reproductivos del parásito. La inducción de estos mecanismos es, en última instancia, el propósito final del protocolo vacunal.

Un segundo punto clave es el proceso de determinación, selección y producción de nuevos antígenos parasitarios que puedan integrarse como candidatos vacunales en formulaciones multivalentes. En la búsqueda de nuevos antígenos adquiere especial relevancia el conocimiento de la expresión antigénica en distintas fases del parásito (NEJ en *Fasciola*); el análisis de epítopos; la implicación de los glicanos en los procesos de reconocimiento antigénico y el papel de las vesículas extracelulares

en esas interacciones celulares. En este proceso resulta básico la disponibilidad de nuevos sistemas de expresión y modificación post-translacional, que, al igual que todos demás campos indicados, tienen su fundamento en las posibilidades que han ofrecido la aplicación de las "omicas" a las relaciones parásito-hospedador.

Otros puntos de interés lo constituyen las nuevas plataformas de administración vacunal, combinando moléculas antigénicas con adyuvantes; la modelización matemática de los procesos vacunales en el sistema epidemiológico de la parasitación; las interacciones vacunales con procesos infecciosos (toxoplasmosis, tuberculosis) y con parámetros productivos; la integración de la vacunación con otros elementos de control parasitario y de manejo de explotaciones y sistemas productivos y los estudios socioeconómicos que garanticen la viabilidad y sostenibilidad de las estrategias integradas de control.

Se hace necesario para todo ello el impulsar un modelo de investigación multidisciplinar, transversal e integrador, que aborde los múltiples aspectos a los que hay que atender para disponer de vacunas realmente aplicables en las explotaciones ganaderas.

Present and future options of parasite control.

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Infections caused by gastrointestinal nematodes (GIN) are one of the most important diseases in grazing ruminants in temperate regions of the world. GIN infections have been regularly controlled with anthelmintic drugs due to their good therapeutic profiles including broad-spectrum of action, good tolerability and low costs. However, these drugs were used in the wrong way for decades -overused, misused, or applied incorrectly- causing the appearance of anthelmintic resistance.

Therefore, new alternatives that combine the use of anthelmintic drugs with methods that are more sustainable in the long term are currently under research. In this sense, one of the main lines of research is the discovery of new drugs, including the development of a methodology that allows large-scale testing of a large number of compounds.

The discovery of new molecules, the identification of natural compounds from plants, or the repurposing and reformulation of known drugs, are the main strategies for the development of new anthelmintic drugs. However, given that every time a drug is marketed there is a risk of resistance developing, the focus should also be on methods that avoid the excessive administration of drugs, such as the use of vaccines or the breeding of animals genetically resistant to GIN infection.

On the other hand, there are some studies that are considering an important component of animals that is in direct contact with GIN and therefore could influence the worm burdens, the gastrointestinal microbiome. Our research group has shown that the composition of the microbiome could be another factor influencing the development of resistance to GIN infection.

Therefore, the use of probiotics with prophylactic or therapeutic application could become a new alternative for the control of GIN infections.