Repurposing of a human antibody-based microarray to explore conserved components of the signalome of the parasitic nematode *Haemonchus contortus*

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**Abstract**

**Background:** Gaining insight into molecular signalling pathways of socioeconomically important parasitic nematodes has implications for understanding their molecular biology and for developing novel anthelmintic interventions.

**Methods:** Here, we evaluated the use of a human antibody-based microarray to explore conserved elements of the signalome in the barber’s pole worm *Haemonchus contortus*. To do this, we prepared extracts from mixed-sex (female and male) adult worms and third-stage larvae (L3s), incubated these extracts on the antibody microarray and then measured the amounts of antibody-bound proteins (‘signal intensity’).

**Results:** In total, 878 signals were classified into two distinct categories: signals that were higher for adults than for larvae of *H. contortus* (n = 376), and signals that were higher for larvae than for adults of this species (n = 502). Following a data-filtering step, high confidence (‘specific’) signals were obtained for subsequent analyses. In total, 39 pan-specific signals (linked to antibodies that recognise target proteins irrespective of their phosphorylation status) and 65 phosphorylation-specific signals were higher in the adult stage, and 82 pan-specific signals and 183 phosphorylation-specific signals were higher in L3s. Thus, notably more signals were higher in L3s than in the adult worms. Using publicly available information, we then inferred *H. contortus* proteins that were detected (with high confidence) by specific antibodies directed against human homologues, and revealed relatively high structural conservation between the two species, with some variability for select proteins. We also in silico-matched 763 compound structures (listed in the DrugBank and Kinase SARfari public databases) to four *H. contortus* proteins (designated HCON_00005760, HCON_00079680, HCON_00013590 and HCON_00105100).

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Conclusions: We conclude that the present antibody-based microarray provides a useful tool for comparative analyses of signalling pathways between/among developmental stages and/or species, as well as opportunities to explore nematocidal target candidates in H. contortus and related parasites.

Keywords: Signalling, Antibody microarray, Repurposing antibodies, Kinases, Phosphorylation

Background
The availability of genomes, transcriptomes and proteomes for the barber’s pole worm (Haemonchus contortus) [1–5]—one of the most pathogenic nematodes of ruminants worldwide [6]—provides a solid foundation for detailed explorations of molecular pathways and processes in this nematode. Haemonchus contortus is representative of many species of order Strongylida, a large order of socioeconomiclly important parasitic roundworms (nematodes). Of particular significance in this context are signalling pathways, because of their crucial roles in a wide range of physiological and developmental processes. Many pathways are regulated by protein kinases, which are enzymes (transferases) that phosphorylate a substrate by transferring a phosphoryl group from an energy-rich molecule, such as adenosine triphosphate (ATP), to a target protein [7]. These kinases are classified into nine key groups and numerous families/subfamilies based on sequence similarity in their catalytic domains and the presence of accessory domains (cf. [8]). Despite the elevation of H. contortus to a ‘model organism’, much work remains to be done to create critical resources and reagents, such as antibody and nucleic acid probes, to identify and characterise key components of signalling pathways and processes.

The lack of commercially available antibodies against proteins from organisms that are not used as classical research models (e.g. mammalian cells, yeast, Drosophila and Caenorhabditis) is a hindrance to research. Consequently, research groups in smaller fields are often forced to generate their own antibodies to study particular proteins of interest, and this precludes system-wide comparisons of signalling proteins between/among developmental stages and/or species, as well as opportunities to explore nematocidal target candidates in H. contortus and related parasites.

Methods
Procurement of H. contortus
Haemonchus contortus (Haecon-5 strain) was maintained in experimental sheep [13], in accordance with institutional animal ethics guidelines (approval permit no. 1714374; The University of Melbourne). Helminth-free Merino sheep (males; 8 months of age) were inoculated orally with 7000 L3s of H. contortus. Faecal samples containing H. contortus eggs were collected every day from 21 days following inoculation. These samples were incubated at 27 °C for 1 week to produce L3s [14]. Larvae were then harvested, sieved through two layers of nylon mesh (pore size: 20 μm; Rowe Scientific PTY, Ltd., Minto, NSW, Australia) to remove debris and dead larvae, washed 5 times in H2O (volume), pelleted by centrifugation at 600 g (5 min) and, following removal of supernatant, frozen at − 80 °C. Adult worms (female and male) were collected from the abomasum of an infected sheep 28 days following inoculation with L3s, washed 5 times in 50-ml volumes of physiological saline, pelleted and then frozen at − 80 °C until use.

Kinexus antibody microarray analysis
The KAM 900P antibody microarray kit (Kinexus Bioinformatics Corp.) was utilised. The KAM-900P chip uses 878 distinct antibodies to protein kinases and other cell signalling proteins; of these antibodies, 265 are pan-specific and 613 are phosphosite-specific. The antibodies
are positioned on a Nexterion-P 3D matrix-coated glass slide, with duplicate spots in two distinct chambers to accommodate two separate samples. Signal detection is performed using a single, non-competitive dye. Cryopreserved *H. contortus* adults (male and female, in an approx. 50/50 mix) and cryopreserved L3s were separately subjected to freeze–thawing cycling in liquid nitrogen and homogenised in physiological saline (pH 7.4). The resultant cellular debris was removed from each sample by centrifugation (10,000 g), and proteins were extracted according to the manufacturer’s instructions to ensure that protease and phosphatase activity was minimised. The samples were loaded onto the microarray chambers for analysis using equal protein concentrations (2 mg/ml; 100 µg of protein for each sample). Following sample loading onto the microarray, the scanning and preliminary readout of the microarray were conducted by the Kinexus Bioinformatics Corp.

Microarray signals that were defined as “low intensity” as well as those with “high error” were removed from this analysis because they represented unreliable leads. Low-intensity signals were defined as signals where the globally normalised L3 signal and the globally normalised adult signal were both <1000 units, consistent with the manufacturer’s recommendations for this antibody-based microarray system. High error signals were defined as signal differences, for which the percentage error of the L3 signals technical duplicates + the percentage signal error for the adult technical duplicates was greater than the magnitude of the percentage difference between the two developmental stages used in this study.

Structure modelling and comparison of kinases and in silico-matching to chemical compounds in public data bases

The program AlphaFold (v2.0) [15] was used to predict the three-dimensional structures of proteins inferred to have differential signals to L3 or the adult stage of *H. contortus* in the antibody-based microarray. Proteins >2500 residues in length were excluded due to technical limitations (relating to the central processing unit [CPU], graphic processing units [GPU] and/or random access memory [RAM]). The program TM-align [16] was used to structurally align homologous sequences between *H. contortus* and human (in a pairwise manner); a structural similarity was expressed as a TM-score, with a score of >0.5 indicating that two structures are similar and related, and a score of <0.2 indicating that they are unrelated. Structures were compared and displayed using the program UCSF ChimeraX [17]. The phosphosites and/or structural alignment of *H. contortus* orthologues were studied and displayed using the PyMOL molecular graphics system v2.5 [18].

The molecules for which antibody-based microarray signals were (1) higher in the *H. contortus* adult than in the L3s and (ii) matched a human homologue, were matched to sequences in the Kinase SARfari [19] and DrugBank v.4.3 [20] databases using PSI-BLAST v.2.2.26+, employing an E-value cut-off of 10^-30 [8]. Chemicals in the Kinase SARfari database were considered if they met the rule-of-five [21] and were predicted to be “medicinal chemistry-friendly”.

**Results**

To determine which human antibodies available in the KAM900 antibody microarray bound to *H. contortus* proteins and whether adult worms and larvae displayed different sets of signals, we prepared samples of mixed-sex (male and female) adult worms and L3 separately, and incubated the samples on the antibody microarray (one sample per chamber; see Methods section for details). The resultant 878 signals were sorted into two categories: signals that were higher in adults than in larvae (n=376), and signals that were higher in larvae than in adults (n=502). To establish which signals were reliable and warranted further investigation, two signal filtering steps were undertaken for the dataset; these removed signals with either low intensity or high error (see Methods section for details). The signal filtering step reduced the number of signals for further analyses to 39 pan-specific signals (linked to antibodies that recognise their target proteins irrespective of their phosphorylation status), 65 phosphorylation-specific signals which were higher in adult *H. contortus*, and 82 pan-specific signals and 183 phosphorylation-specific signals which were higher in the L3s (Table 1; “Total” vs. “Reliable”). A complete table of all signals measured is available (Additional file 1: Table S1; Additional file 2: Table S2).

The signals classified as reliable were explored further. To aid visual interpretation of log2-fold change between the two development stages of *H. contortus*, we represented the signal differences between the samples in a scatter plot (Fig. 1). For each of the comparisons conducted here, there were several notable signals based on the magnitude of their fold changes, including those linked to the molecules ribosomal protein S6 kinase beta-1 (pP70 S6K), Cofilin 1, cytosolic tyrosine kinase (CSK), SHIP2 (SH2-containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase) and ERK4 (mitogen-activated extracellular signal-regulated kinase 4). Following signal filtering, there were notably more signals that were higher in L3s than in the adult stage (2-fold more for pan-specific signals, 3-fold more for phospho-specific signals). This finding is consistent with the increased signalling activity expected in developing (vs. terminally differentiated) tissues. Importantly, we identified
which antibodies were highly likely to recognise a genuine *H. contortus* homologue of the human target protein. This was made possible through the availability of the antibody epitopes and uniport ID for each human protein targeted on the microarray. Using publicly available genomic and transcriptomic data sets from Parasite WormBase [2, 22], we identified nucleotide sequences coding for the proteins represented by high signals in the microarray (Table 2). Manual curation of the protein sequences identified distinct homologues between human and *H. contortus* (Additional file 3: Figure S1). Only protein sequence matches with an identity/E-value of $10^{-5}$ and fold change of $>2.0$ were included. The complete, matched list of antibody signals to the *H. contortus* proteins is provided in Table 1. Subsequent comparative modelling of these proteins revealed, overall, relatively high structural conservation between the two species, although some sub-structural elements within some proteins (e.g. HCON_00125210 and HCON_00080620; Additional file 3: Fig. S1) were variable (<50% on colour scale). In silico-matching to chemicals in public databases (i.e. DrugBank and Kinase SARfari databases) revealed 763 compounds that matched four *H. contortus* proteins (HCON_00005760, HCON_00079680, HCON_00013590 and HCON_00105100; Table 3; Additional file 4: Table S3; Additional file 5: Table S4).

**Discussion**

Gaining insight into molecular signalling pathways of socioeconomically important parasitic nematodes has major relevance for developing new interventions against the diseases that these worms cause in animals and humans [23–25], because it should be possible to identify targets in these pathways for the design of new anthelmintics. Given the major adverse impact of diseases caused by these worms, this focus is critical. Anthelmintic treatment is a key component of most parasite control programmes, but resistance to most available anthelmintics (cf. [26]). At the present time, the small number of classes of anthelmintics available [27, 28] and the problem of widespread anthelmintic resistance [29] demand concerted efforts to discover new anthelmintic drugs with novel mechanisms/modes of action. Thus, some of the proteins identified here in the adult stage of *H. contortus* (which is the blood-feeding and pathogenic stage of the nematode) could be promising target candidates to evaluate, as the druggability of protein kinases is well-established [30], with >70 kinase inhibitors on the market and >150 in development.

| Table 1 Summary of results for the antibody microarray signal filtering and lead signal determination for protein samples from adult and third-stage larvae of *Haemonchus contortus* |
|---------------------------------|-----------------|-----------------|
| **Microarray signals**          | **Signals higher in adults** | **Signals higher in L3** |
| **Total pan-specific**          | 114             | 151             |
| **Low intensity**               | 34              | 12              |
| **High error**                  | 41              | 57              |
| **Reliable**                    | 39              | 82              |
| **Total phospho-specific**      | 262             | 351             |
| **Low intensity**               | 89              | 51              |
| **High error**                  | 108             | 117             |
| **Reliable**                    | 65              | 183             |

Fig. 1 Dot plot of antibody microarray results separated according to which signals were higher in the adult stage or L3 of *Haemonchus contortus*. Signals represent the log2-fold change between the adult and L3 samples compared. Left-side of dot plot: signals for the pan-specific and phosphorylation-specific antibodies on the array that were higher in the adult stage than in L3. Right-side of dot plot: signals for the pan-specific and phosphorylation-specific antibodies on the array that were higher in L3 than in the adult stage. The largest log2-fold change-associated signals are annotated above each plot. Signals which were flagged as low intensity and affected by high error have not been included here (signal filter strategy used is described in the Methods section). L3, Third-stage larvae; also see Abbreviation list
The set of kinase inhibitors predicted here \( (n=763) \), inferred to match three homologues in adults and in L3 of *H. contortus* (cf. Table 2), could be a useful starting point for targeted compound screening on *H. contortus*. Thus, a subset of these compounds could be selected as drugs, depending on the purchase cost, availability, chemical properties, safety and/or prior use(s), and tested for anthelmintic effects in a recently established, automated, whole-worm motility screening assay [15]. This step would be followed by a hit-to-lead phase, in which structural analogues would be synthesised and studied to establish structure–activity relationships, with the aim to maximise nematocidal effect and minimise binding to mammalian (host) homologues. Subsequent work could then focus on intestinal absorption, distribution, metabolism, excretion and toxicity assessments.

### Table 2
Summary of *H. contortus* proteins for which antibody-based microarray signals were high in the adult \( (n=18) \) versus the third-stage larvae \( (n=17) \) and had a matched human homologue

| Human target protein | Phospho-site (Human) | Phosphosite sequence | Uniprot ID | Fold-change | H. contortus homologue | TM-score | Conserved phospho-site* | Stage |
|----------------------|----------------------|----------------------|------------|-------------|------------------------|----------|------------------------|-------|
| p70 S6K              | T252                 | TL(pS)PI             | P23443     | 31.62       | HCON_00079680          | 0.71201  | Yes                    | Adult |
| ELK1                 | S383                 | MA(pS)GVA            | P19419     | 2.61        | HCON_00127560          | 0.3449   | No                     | Adult |
| eIF4E                | S209                 | KSG(pT)TKK           | P06730     | 2.38        | HCON_00133690          | 0.81774  | Yes                    | Adult |
| Integrin a4          | S1021                | RRD(pS)WSY           | P13612     | 2.23        | HCON_00080620          | 0.50131  | Yes                    | Adult |
| Histone H3           | S28                  | ARK(pS)APS           | P84243     | 2.21        | HCON_00065870          | 0.96784  | Yes                    | Adult |
| Histone H3           | T3                   | VTH(pT)FGC           | P84243     | 2.17        | HCON_00065870          | 0.96784  | Yes                    | Adult |
| SYK                  | Y323                 | AR(pT)KQT            | P43405     | 2.14        | HCON_00013780          | 0.53671  | No                     | Adult |
| Crystallin aB        | S19                  | PFH(pS)PSR           | P02511     | 2.10        | HCON_00114930          | 0.49117  | Yes                    | Adult |
| Histone H2B          | S14                  | SAPAPKKG(pS)KK       | P33778     | 2.09        | HCON_00026210          | 0.74892  | Yes                    | Adult |
| PI3K                 | Pan-specific         | na                   | P27986     | 4.68        | HCON_00017820          | 0.33794  | na                     | Adult |
| CSK                  | Pan-specific         | na                   | P41240     | 4.37        | HCON_00013590          | 0.69703  | na                     | Adult |
| Arrestin b           | Pan-specific         | na                   | P49407     | 2.39        | HCON_000167390         | 0.87674  | na                     | Adult |
| Crystallin aB        | Pan-specific         | na                   | P02511     | 2.37        | HCON_00114930          | 0.49117  | na                     | Adult |
| Bmx                  | Pan-specific         | na                   | P51813     | 2.32        | HCON_00026740          | 0.47372  | na                     | Adult |
| HSP90a/b             | Pan-specific         | na                   | P07900     | 2.27        | HCON_00036990          | 0.92043  | na                     | Adult |
| CDK7                 | Pan-specific         | na                   | P50613     | 2.15        | HCON_00005760          | 0.9175   | na                     | Adult |
| CDK1                 | Pan-specific         | na                   | P06493     | 2.08        | HCON_00101500          | 0.95318  | na                     | Adult |
| IkBα                 | Pan-specific         | na                   | P25963     | 2.04        | HCON_00125210          | 0.20125  | na                     | Adult |
| SgK56                | Y635                 | PNA(pY)OJNL          | Q9H792     | 2.30        | HCON_00028850          | 0.23692  | No                     | L3    |
| YSK1                 | T174                 | KRN(pT)FVG           | O00506     | 2.18        | HCON_00162520          | 0.64192  | Yes                    | L3    |
| InsR (IR)            | Y1189                | ETD(pY)YRK           | P06213     | 2.16        | HCON_00065690          | 0.51222  | Yes                    | L3    |
| MAPKAPK2             | Y225 + T226          | PC(pY)(pT)PYV        | P49137     | 2.15        | HCON_00101270          | 0.83651  | Yes                    | L3    |
| ErbB3 (HER3)         | Y1307                | HV(pY)ARL           | P21860     | 2.09        | HCON_00013870          | 0.35282  | No                     | L3    |
| B-Raf (RafB)         | S729                 | RSA(pS)PFS           | P15056     | 2.04        | HCON_00111880          | 0.41322  | Yes                    | L3    |
| VIM                  | Y117                 | FAN(pY)IDK           | P08670     | 2.04        | HCON_00059550          | 0.41927  | Yes                    | L3    |
| ERK4 (MAPK4)         | Pan-specific         | na                   | P31152     | 2.89        | HCON_00091920          | 0.64358  | na                     | L3    |
| JAK2                 | Pan-specific         | CWNVNVQRPS-FRDLA     | O60674     | 2.34        | HCON_00109060          | 0.29177  | na                     | L3    |
| MKK3                 | Pan-specific         | CTDIAFVKELGEDS       | P46734     | 2.24        | HCON_00105100          | 0.84979  | na                     | L3    |
| ASK1                 | Pan-specific         | na                   | Q99683     | 2.18        | HCON_00040310          | 0.64569  | na                     | L3    |
| WNK1                 | Pan-specific         | CLETKAVGMSDGRL       | Q9H4A3     | 2.14        | HCON_00107110          | 0.29247  | na                     | L3    |
| RET                  | Pan-specific         | CCRPVAFDSDKLEKM      | P07949     | 2.11        | HCON_00187950          | 0.3504   | na                     | L3    |
| B-Raf (RafB)         | Pan-specific         | CSDDWEPDQITVGQ       | P15056     | 2.10        | HCON_00111880          | 0.41322  | na                     | L3    |
| EphA1                | Pan-specific         | na                   | P21709     | 2.05        | HCON_00042040          | 0.32568  | na                     | L3    |
| MKK3                 | Pan-specific         | CAERMSYELMEHPFF      | P46734     | 2.02        | HCON_00105100          | 0.84979  | na                     | L3    |
| VEGFR2 (KDR)         | Pan-specific         | CILQPDSGTTLSSPPV     | P35968     | 2.01        | HCON_00185500          | 0.26013  | na                     | L3    |

*na* not available

* The structural conservation of phospho-sites is shown in Additional file 3: Figure S1
Given that *H. contortus* shares many orthologues with other clade V nematodes, the current work provides a starting point for the identification of novel drug targets in a range of socioeconomically important parasitic nematodes of human health importance (e.g. hookworms *Necator americanus* and *Ancylostoma duodenale*) and veterinary significance (e.g. species of *Trichostrongylus, Teladorsagia, Ostertagia, Cooperia* and many more). Moreover, the free-living nematode *Caenorhabditis elegans* (clade V), arguably the best characterised multicellular organism, could be used as a complementary or surrogate tool for the validation of targets and the mechanisms/modes of action of optimised compounds and for the prediction of resistance development in nematodes.

**Conclusion**

In conclusion, the present antibody-based microarray has opened the door to fundamental investigations of signalling pathways in free-living and parasitic nematodes, and also between distinct developmental stages of these worms, and provides opportunities for the discovery and exploration of critical targets and new anthelmintics.

**Abbreviations**

ATP: Adenosine triphosphate (ATP); CSK: 50-KDa cytosolic tyrosine kinase; ERK4: Mitogen-activated extracellular signal-regulated kinase 4; L3s: Third-stage larvae; p70 S6K: Ribosomal protein S6 kinase beta-1; SHIP2: SH2-containing phosphatidylinositol 3,4,5-trisphosphate 5-phosphatase.

**Supplementary Information**

The online version contains supplementary material available at https://doi.org/10.1186/s13071-022-05400-w.

**Additional file 1:** Table S1. Complete list of signals recorded as being higher in the adult stage of *Haemonchus contortus* than in the larval stage (L3)—in the Kinexus antibody microarray experiment.

**Additional file 2:** Table S2. Complete list of signals recorded as being higher in the larval stage (L3) of *Haemonchus contortus* than in the adult stage—in the Kinexus antibody microarray experiment.

**Additional file 3:** Figure S1. Three-dimensional structural models for *Haemonchus contortus* proteins with high antibody-based microarray signals, compared with their orthologues in *Homo sapiens* in a pairwise manner. Conserved regions are in pink, and divergent ones in green. The phosphosites in the protein sequence and structure is indicated in green and box, respectively.

**Additional file 4:** Table S3. Chemicals in the DrugBank database associated with the detected *Haemonchus contortus* proteins in the present study.

**Additional file 5:** Table S4. Chemicals in KinaseSARfari database associated with the detected *Haemonchus contortus* proteins in the present study.

**Acknowledgements**

Not applicable.

**Author contributions**

Conceived and designed the study and supervised the project: CD, JA and RBG. Undertook the study and data analysis: JA, TW and GM. Contributed to analysis using various tools: YZ and NDY. Contributed to the interpretation of findings and drafting of the manuscript: JA, TW, CD and RBG. All authors read and approved the final manuscript.

**Funding**

This study was supported by the Australian Research Council (ARC), Yourgene Health Singapore and the Australian National Health and Medical Research Council (NHMRC; APP2003712).

**Availability of data and materials**

All data generated or analysed during this study are included in this article.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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Received: 17 May 2022   Accepted: 14 July 2022
Published online: 30 July 2022

**Table 3** Predicted kinase inhibitors that matched four *H. contortus* proteins representing two developmental stages—adult and third-stage larvae

| Protein encoded by *Haemonchus contortus* gene | Developmental stage | Number of compounds in DrugBank databasea | Number of compounds in Kinase SARfari databasea |
|-----------------------------------------------|---------------------|------------------------------------------|-----------------------------------------------|
| HCON_00005760                                | Adult               | 2                                        | 0                                             |
| HCON_00079680                                | Adult               | 4                                        | 0                                             |
| HCON_00013590                                | Adult               | 12                                       | 735                                           |
| HCON_00105100                                | L3                  | 10                                       | 0                                             |

* Individual compound codes are given in Additional file 4: Table S3; Additional file 5: Table S4, respectively.
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