Analysis of the protein repertoire revealed in the medium during prolonged incubation of *Trichinella pseudospiralis* muscle larvae *in vitro*

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Summary. Proteins accumulated in the medium during prolonged incubation of *Trichinella pseudospiralis* ISS7626 (domestic pig *Sus scrofa* (Kamchatka)) muscle larvae were analysed by LC-MS/MS. A wide repertoire of excretory-secretory and somatic proteins ranging from 72 to 238 was revealed in samples, correlating with a decrease in the reproduction potential of the larvae, which remained invasive. A gene ontology annotation shows that most of revealed proteins are nucleases, proteases or their regulators; enrichment in extracellular compartment-related proteins and proteins involved in cell attachment predominated. Sixty-nine of the revealed proteins were permanently present in all analysed samples, which indirectly confirm that most of the larvae were alive during the incubation period. Orthologous clustering demonstrates that most of the permanently present proteins are *T. pseudospiralis* strain-specific. High level of sequence identity within clusters and frequent presence of paralogous genes within cluster point to the recent emergence of these orthologous groups and rapid evolving of proteins during adaptation to wide range of hosts.

Key words: excretory-secretory proteins, gene ontology, host-parasite interactions, LC-MS/MS, orthologous clustering, trichinellosis.

Trichinella pseudospiralis is a pathogenic nematode, the causative agent of a zoonotic disease named trichinellosis in humans and animals (Gottstein *et al.*, 2009). *T. pseudospiralis* is one of the three species whose larvae do not encapsulate after muscle cell differentiation, and the only one infecting both mammals and birds (Pozio & Zarlenga, 2013; Odoevskaya *et al.*, 2018a; Krapivin & Odoevskaya, 2019; Seryodkin *et al.*, 2020). Up to now, it was found in 18 species of mammals and eight species of birds (La Rosa *et al.*, 2001; Pozio, 2016). Even more than 40 years after discovery of *T. pseudospiralis* its epidemiology remains obscure.

In humans, the disease is characterised by severe pathological manifestations and a long period of recovery even with a weak intensity of invasion (Posio, 2016). The clinical manifestations of this disease are related to the cytological changes in the host myocytes involving satellite cells, leucocytes, neutrophils and macrophages (Britov, 1982). Under the influence of excretory-secretory (E-S) products of *Trichinella* muscle larvae, these cells merge into single simplasts each retaining a functioning sarcoplasm with large nuclei. Thus, the parasite provides itself with optimal conditions for a long-term intracellular life in a host-parasite complex (Garkavi, 2007). Throughout its life, larvae secrete various substances including proteins that cause intense alterations in the host's muscle fibres (*i.e.*, destruction, melting and degeneration) with the formation of foci of cellular infiltration and regeneration (Britov, 1997; Garkavi, 2007). In addition, the mass death of *Trichinella* in the host body leads to hypersensitivity, severe allergic reactions, and provokes the development of edematous syndrome and muscle myalgia (Britov, 1982). Epidemic studies conducted in 2014-2018 in the Far Eastern region of Russia revealed the presence of *T. pseudospiralis* distributed intermittently, registered in wild (Bengal cat, fox,

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brown bear and raccoon dog) and domestic animals (Britov, 1997; Odoevskaya *et al.*, 2018a; Seryodkin *et al.*, 2020). The isolate of *T. pseudospiralis* ISS7626 (domestic pig *Sus scrofa* (Kamchatka), originating from this region and used in our work, deserves special attention due to its high virulence, wide distribution and hence potential health threat to populations. Up to now, it is practically unexplored, and proteins released by this nematode and important for the establishment and further long-term existence of host-parasite complex are not studied. Analyses of E-S and somatic proteins of other isolates of *T. pseudospiralis* made earlier revealed important immunogenic proteins and point to species specificity and the differential expression of E-S proteins in geographically distant isolates of *T. pseudospiralis* (Robinson *et al.*, 2007; Wu *et al.*, 2013; Wang *et al.*, 2017, 2021; Odoevskaya *et al.*, 2018b; Somboonpatarakun *et al.*, 2018).

Investigation of excretory-secretory proteins characterising *Trichinella* spp. in general and *T. pseudospiralis* in particular is usually conducted on products obtained after 18-20 h of muscle larvae (ML) incubation in a nutrient medium (Robinson *et al.*, 2007; Bien *et al.*, 2013; Wang *et al.*, 2013; Wang *et al.*, 2017). This model does not take into account that the total repertoire of proteins, released by the larvae into surrounding muscle tissues *in vivo*, could be much more complicated and can include not only E-S proteins, but also somatic proteins, originating from dead larvae. These proteins can also affect the host cells during infection or treatment of the disease. We believe that without analysing the total repertoire of proteins released by the ML during incubation *in vitro* it is impossible to judge with confidence which ones are important for the processes taking place *in vivo*. There are some observations pointing to changes in morphology of *T. pseudospiralis* ML during prolonged incubation of over 18 h (Robinson *et al.*, 2007), which could reflect loss of viability by some of the larvae. However, no analysis of the protein repertoire was done and nobody studied if any correlation exists between the number of revealed proteins and the percentage of surviving/dead larvae.

The aim of the present work was to obtain and analyse the total repertoire of proteins accumulated in the nutrition medium during incubation of ML (*T. pseudospiralis* ISS7626) *in vitro*, prolonged over 18 h and to investigate changes in this repertoire with time. Mass spectrometric analysis (LC-MS/MS) was used to reveal proteins obtained at 24, 48 and 72 h of *in vitro* incubation, when most of the larvae still maintain morphological integrity and physiological activity. Gene ontology (GO) enrichment analysis was used to categorise the revealed proteins according to their molecular function, biological process and cell localisation. Proteins permanently present (PP proteins) during the whole investigated period (24-72 h) and those that appeared to be specific for each period of incubation were identified. Determination of orthologous groups (OG) was applied to PP proteins to identify their species and strain specificity.

MATERIAL AND METHODS

Parasites and experimental animals. The strain of capsule-free trichinella *T. pseudospiralis* ISS7626 was originally isolated from the muscles of a domestic pig *S. scrofa* in 2015 during an investigation of an epidemic outbreak of trichinellosis in Kamchatka. To establish the taxonomic affiliation of this strain, a 847 bp region of mitochondrial DNA corresponding to subunit I (COXI) of the cytochrome c oxidase gene was sequenced (assigned GenBank access number MH257740). The affiliation of this strain to the species *T. pseudospiralis* was confirmed by the International Reference Center for Trichinosis ITRC (assigned code is ISS7626). The viability and virulence of this strain was maintained by successive passages on birds (quails) and laboratory rodents (white rats and mice).

Collection of larvae. The required number of same-age infective muscle larvae was obtained as described previously (Odoevskaya *et al.*, 2018b).

Test on biological activity of the ML. Muscle larvae, obtained after 24, 48 and 72 h of incubation in DMEM (Dulbecco's Modified Eagles Medium; PanEco Ltd., Russia) were used for infection of mice. After 1 month, an intensity of infection was determined – expressed as number of *Trichinella* larvae per g of minced tissues. The reproduction potential was calculated by dividing the total number of larvae extracted from obtained minced tissues by the number of larvae introduced during infection, separately for each experimental time period.

Test on microbial contamination. To confirm the absence of microbial contamination aliquots of the DMEM medium were selected for sowing on solid agar medium at the end of each period of larvae incubation – 24, 48 and 72 h; aliquots were also analysed by LC-MS/MS against selected bacterial protein data presented in UniProt data base.

Preparation of samples for LC-MS/MS analysis. Processing of obtained culture media and preparation of samples for LC-MS/MS analysis was done as described previously (Odoevskaya *et al.*, 2018b).

Liquid chromatography and mass spectrometry. The LC-MS/MS analysis was carried out as described previously (Odoevskaya *et al.*, 2018b; Fefilova *et al.*, 2020). Each sample was analysed in three technical replicates and not less than two biological replicates.

Data analysis. The analysis of the qualitative MS/MS based proteome of *Trichinella* E-S samples was performed using PEAKS Studio 8 build 20160908 software (Ma *et al.*, 2003). Peptide lists generated by the PEAKS Studio were searched against *T. pseudospiralis* database (http://parasite.wormbase.org/), (for identification of proteins and corresponding accession numbers ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/001/447/645/GCA_001447645.1_T4_ISS176_r1.0 was used) with cysteine carbamidomethylation as a fixed modification and N-terminal acetylation and methionine oxidations as variable modifications. The false discovery rate (FDR) for peptide-spectrum matches was set to 0.01 and was determined by searching a reverse database. Enzyme specificity was set to trypsin, and a maximum of three missed cleavages were allowed in the database search. Peptide identification was performed with an allowed initial precursor mass deviation up to 10 ppm and an allowed fragment mass deviation of 0.05 Da. Reliably defined was considered as a protein identified by not less than by four unique peptides.

GO enrichment analysis. GO annotations for *T. pseudospiralis* were taken from UniProt database using custom python script and requests python module. Enrichment analysis was carried out for the list of proteins reliably detected during this study. Statistical testing and GO graph visualisation was done with top.GO R package (Alexa & Rahnenfuhrer, 2022) following the author's guidelines. Significance threshold for reported results was set to 0.01 (adjusted *P*-value, exact Fisher test).

Clustering and selection analysis. Clusters of orthologous genes were determined using cd-hit v.4.7. (Li & Godzik, 2006) using annotated protein sequences for *Trichinella britovi*, *T. murrelli*, *T. nativa*, *T. nelsoni*, *T.papuae*, *T. patagoniensis*, *T. pseudospiralis* ISS13, *T. pseudospiralis* ISS141, *T. pseudospiralis* ISS176, *T. pseudospiralis* ISS470, *T. pseudospiralis* ISS588, *T. spiralis*, *T.* sp. T8, and *T. zimbabwensis* downloaded from WormBase ParaSite (version WBPS14) and 69 E-S protein sequences of *T. pseudospiralis* ISS7626. Orthologous groups that contain one of 69 *T. pseudospiralis* ISS7626 proteins are listed in Table 9 (Supplemental material). Best blast hit annotation was done with stand-alone ncbi-blast suite v. 2.3.0+ (Camacho *et al.*, 2009) with blast program against nr protein database, e-value cut-off 10^{-6}. Multiple protein-coding codon alignments were generated by aligning protein sequences with mafft v. 7.305 (Katoh & Standley, 2013) and refining final codon alignments with custom python script. Selective pressure (ω value) for each alignment position was calculated with ETE3 toolkit (Huerta-Cepas *et al.*, 2016), M2 model from Codeml (Yang, 2007) as tested against M1 as the hypothesis; sites with $\omega > 1$ were counted.

RESULTS

Viability of the ML at different incubation times. We analysed the morphology of the muscle larvae of *T. pseudospiralis* (ISS 7626) during prolonged *in vitro* incubation in a liquid medium DMEM. We did not find any confirmation of detachment of the cuticle, or any other signs of changed morphology in the majority of ML up to 72 h of incubation. Testing of biological activity showed that larvae collected after 24, 48 and 72 h of incubation *in vitro* maintained biological activity and were able to infect mice, though the reproduction potential (intensity of infection) decreased with time (Table 1).

Table 1. Number of proteins revealed in the culture medium at different incubation times *versus* magnitude of the reproduction potential (intensity of infection of mice) and degree of invasiveness of *Trichinella pseudospiralis* muscle larvae.

Duration of muscle larvae incubation in hours	24	48	72
Reproduction potential	26	18.3	10.5
The degree of invasiveness in %	100%	100%	100%
Number of proteins revealed in the culture medium	72	155	238

Considering this, to obtain a total repertoire of E-S and somatic proteins accumulated during incubation of *T. pseudospiralis* (ISS7626) ML *in vitro* and to trace possible changes in protein repertoire we used three time intervals – 24, 48 and 72 h.

Table 2. Proteins revealed at both 48 and 72 h of *Trichinella pseudospiralis* (ISS7626) muscle larvae incubation.

No.	Accession*	Proteins and groups of proteins
1	KRZ38578.1	Enolase, partial
2	KRZ42230.1	Protein disulfide-isomerase 2
3	KRZ41997.1	Cell-death-related nuclease 7
4	KRZ34658.1	Myoglobin, partial
5	KRZ37225.1	14-3-3-like protein 2
6-8	KRZ32644.1, KRZ32646.1, KRZ32645.1	Golgi apparatus protein 1-like prot partial
9-13	KRZ36272.1, KRZ36270.1, KRZ36271.1, KRZ36269.1, KRZ36268.1	Vitellogenin, partial
14-15	KRZ46361.1, KRZ46360.1	Leukocyte elastase inhibitor C, partial
16	KRZ30674.1	Sulfhydryl oxidase 1
17	KRZ40120.1	Fructose-bisphosphate aldolase 1
18	KRZ37963.1	Phosphoenolpyruv. carboxykinase [GTP]
19	KRZ37229.1	Elongation factor 2
20-26	KRZ37258.1, KRZ41616.1, KRZ41617.1, KRZ37490.1, KRZ31576.1, KRZ38785.1, KRZ36910.1	Hypothetical protein T4C_10006, partial; T4C_1962, T4C_1962, T4C_13146, partial; T4C_10889, partial; T4C_2522, T4C_5945
27	KRZ41279.1	Actin-related protein 2/3 complex subunit 2, partial
28	KRZ43809.1	Glutaredoxin-C8
29-33	KRZ44858.1, KRZ44863.1, KRZ44862.1, KRZ44864.1, KRZ44860.1	Heat shock 70 kDa protein, partial
34	KRZ43867.1	Peroxiredoxin-2, partial
35	KRZ40784.1	Heat shock protein 83, partial
36	KRZ40839.1	Actin-depolymerizing factor1, isoforms a/b, partial
37-38	KRZ43885.1, KRZ43884.1	Transmembrane matrix receptor MUP-4
39	KRZ44280.1	Neural cell adhesion molecule 1
40	KRZ35908.1	Serine protease inhibitor Kazal-type 4, partial
41	KRZ37722.1	Onchocystatin
42	KRZ39503.1	Triosephosphate isomerase
43-44	KRZ39344.1, KRZ39343.1	Nidogen-1
45-46	KRZ38515.1, KRZ38511.1	Cleavage and polyadenylation specificity factor subunit 2
47	KRZ30445.1	OV-16 antigen
48	KRZ40448.1	Superoxide dismutase [Cu-Zn]
49	KRZ45513.1	Dedicator of cytokinesis protein 1
50-52	KRZ33559.1, KRZ33555.1, KRZ33558.1	Papilin
53-54	KRZ42802.1, KRZ42801.1	Collagen alpha-6(VI) chain
55-59	KRZ39082.1, KRZ39074.1, KRZ39083.1, KRZ39078.1, KRZ39075.1	Basement membrane proteoglycan
60	KRZ40747.1	Peptidyl-prolyl cis-trans isomerase B, partial
61	KRZ40618.1	Protein-L-isoaspartate(D-aspartate) O-methyltransferase
62-63	KRZ30232.1, KRZ30230.1	Antigen-like protein, partial
64-66	KRZ35474.1, KRZ35476.1, KRZ35475.1	Glycogen phosphorylase, partial
67	KRZ43531.1	Protein NPC2-like protein
68-69	KRZ44440.1, KRZ44439.1	32 kDa beta-galactoside-binding lectin lec-3, partial
70	KRZ28015.1	Intermediate filament protein B
71	KRZ33676.1	ADP-ribose pyrophosphatase, mitochondrion

* – accession number according to UniProt database – *T. pseudospiralis*.

Number of revealed proteins. To identify proteins accumulated in the incubation medium obtained at 24, 48 and 72 h we have applied LC-MS/MS analysis. A wide repertoire of proteins was revealed, the diversity of which increased with time of incubation: 72 proteins at 24 h, 155 at 48 h and 238 proteins at 72 h of incubation (Table 1).

Dynamics of revealed protein repertoires. Pair-wise comparison of proteins in the medium after 24, 48 and 72 h of incubation enabled us to select those that were specific for certain time periods, but absent at others. We compaired protein repertoires at 24 h *vs* 48 h, 24 h *vs* 72 h, or 48 h *vs* 72 h. Two proteins, a putative nudix hydrolase 6 (a hydrolytic enzyme capable of cleaving nucleoside diphosphates) and a hypothetical protein T4C_5429 were present both at 24 h and 48 h but were not found at 72 h. Only one hypothetical protein T4C_8913 was found both at 24 h and 72 h. Seventy-one proteins were present both at 48 h and at 72 h and not found at 24 h (Table 2).

Analyses of protein repertoires at 24, 48 and 72 h of ML incubation demonstrated that a group of 69 proteins was permanently and invariably represented in all analysed samples and was designated by us as permanently present (PP proteins) (Table 3).

Table 3. Permanently present proteins (PP proteins), revealed in the medium at all incubation times (24, 48 and 72 h) of *Trichinella pseudospiralis* (ISS7626) muscle larvae.

No.	Accession*	Proteins and protein groups
1	KRZ41512.1	Transmembrane protease serine 9
2-32	KRZ41667.1, KRZ30654.1, KRZ45192.1, KRZ39651.1, KRZ30558.1, KRZ38119.1, KRZ43906.1, KRZ41740.1, KRZ38482.1, KRZ38481.1, KRZ40304.1, KRZ41676.1, KRZ42593.1, KRZ45279.1, KRZ38964.1, KRZ39248.1, KRZ45699.1, KRZ24141.1, KRZ30317.1, KRZ37581.1, KRZ45632.1, KRZ42726.1, KRZ33045.1, KRZ46409.1, KRZ24140.1, KRZ44441.1, KRZ35585.1, KRZ42470.1, KRZ26446.1, KRZ43329.1, KRZ32006.1	Hypothetical proteins: T4C_499, T4C_9070, T4C_9969, T4C_6737, T4C_8213, T4C_8699, T4C_6718, partial; T4C_5154, partial; T4C_13673, T4C_13673, T4C_2403, T4C_10263, T4C_8017, T4C_5355, T4C_12478, T4C_10423, T4C_9126, T4C_2777, partial; T4C_10441, T4C_13186, T4C_10093, T4C_6079, T4C_4514, partial; T4C_4663, T4C_88, partial; T4C_2225, T4C_10719, T4C_10402, partial; T4C_10429, T4C_4891, T4C_12851, partial
33-39	KRZ41757.1, KRZ27161.1, KRZ27169.1, KRZ27168.1, KRZ27160.1, KRZ30412.1, KRZ43013.1	ADP-ribose pyrophosphatase, mitochondrial
40-41	KRZ43352.1, KRZ43353.1	RWD domain-containing protein 2B
42	KRZ38469.1	Protein piccolo
43-44	KRZ45847.1, KRZ30749.1	Chymotrypsin-like elastase family member 1
45	KRZ43229.1	Plancitoxin-1
46	KRZ41737.1	GLIPR1-like protein 1, partial
47	KRZ36022.1	5'-nucleotidase
48-49	KRZ36887.1, KRZ36886.1	Poly-cysteine and histidine-tailed protein, partial
50	KRZ36021.1	Snake venom 5'-nucleotidase
51	KRZ44034.1	Deoxyribonuclease-2-alpha, partial
52-53	KRZ37713.1, KRZ37714.1	Tissue-type plasminogen activator
54	KRZ45281	Putative N-acetylglucosamine-6-phosphat deacetylase
55	KRZ43692.1	Carboxylesterase 1E
56	KRZ32113.1	Pancreatic alpha-amylase, partial
57	KRZ30414.1	Mitochondrial-processing peptidase subunit alpha
58	KRZ32125.1	Elongation factor Ts, mitochondrial
59	KRZ26435.1	Myoglobin
60	KRZ38229.1	Chymotrypsinogen B, partial
61	KRZ38606.1	Transforming growth factor
62	KRZ38605.1	Periostin, partial
63	KRZ38348.1	Elongation factor 1-alpha, partial
64	KRZ42649.1	Cyclin-K
65	KRZ42513.1	Poly(U)-specific endoribonuclease -like protein
66-68	KRZ42878.1, KRZ42879.1, KRZ42880.1	Acyl-coenzyme A thioesterase 8, partial
69	KRZ33247.1	Prostasin

* – accession number according to UniProt database – *T. pseudospiralis*.

In addition to 69 PP proteins detected throughout the whole period of investigation (24-72 h) and those found by pair-wise comparisons (24 h *vs* 48 h, 24 h *vs* 72 h, 48 *vs* 72 h), we also found a number of proteins specific and unique for each separate time period and not found in the products of any other one. No proteins were specific to 24 h of incubation, but 13 of them were found only at 48 h and 97 only at 72 h of incubation (Tables 4 & 5), which corresponds in general to an increase in the total number of detected proteins with time.

Table 4. Specific proteins revealed at 48 h of incubation of *Trichinella pseudospiralis* (ISS7626) muscle larvae.

No.	Accession*	Proteins and groups of proteins
1-2	KRZ36785.1, KRZ36784.1	Hypothetical proteins: T4C_247, T4C_247
3	KRZ44859.1	Heat shock cognate 71 kDa protein, partial
4-5	KRZ21765.1, KRZ21766.1	1,5-anhydro-D-fructose reductase
6	KRZ44279.1	Neural cell adhesion molecule 2, partial
7	KRZ44278.1	Neural cell adhesion molecule 1
8-10	KRZ39076.1, KRZ39077.1, KRZ39079.1	Basement membrane proteoglycan
11	KRZ30231.1	Antigen-like protein, partial
12	KRZ36198.1	Nucleoside diphosphate kinase
13	KRZ33674.1	ADP-ribose pyrophosphatase, mitochondrion

* – accession number according to UniProt database – *T. pseudospiralis*.

Table 5. Specific proteins revealed at 72 h of incubation of *Trichinella pseudospiralis* (ISS7626) muscle larvae.

No.	Accession*	Proteins and groups of proteins
1	KRZ36483.1	Spectrin alpha chain
2-9	KRZ44699.1, KRZ44688.1, KRZ44690.1, KRZ44693.1, KRZ44694.1, KRZ44695.1, KRZ44689.1, KRZ44696.1	Protein sidekick-1
10	KRZ45168.1	Glucose-6-phosphate isomerase
11-12	KRZ30762.1, KRZ30763.1	Intermediate filament protein ifa-1
13	KRZ33864.1	Teneurin-a, partial
14-21	KRZ38768.1, KRZ38773.1, KRZ38765.1, KRZ38771.1, KRZ38766.1, KRZ38774.1, KRZ38772.1, KRZ38767.1	Spectrin beta chain, partial
22	KRZ43553.1	Rab GDP dissociation inhibitor alpha, partial
23	KRZ29798.1	E3 ubiquitin-protein ligase makorin-1, partial
24-26	KRZ40903.1, KRZ40905.1, KRZ40904.1	Putative integrin beta-like protein, partial
27-28	KRZ28240.1, KRZ28241.1,	Disorganized muscle protein 1
29-31	KRZ34625.1, KRZ34624.1, KRZ34627.1	Microtubule-actin cross-linkngfactor1, part
32	KRZ34015.1	Paramyosin
33-36	KRZ44645.1, KRZ44648.1, KRZ44647.1, KRZ44646.1	Angiotensin-converting enzyme
37	KRZ27525.1	Protein DJ-1
38	KRZ31041.1	Putative splicing factor, arginine/serine-rich 7
39	KRZ45314.1	Putative tyrosinase-like protein tyr-3, partial

Table 5 (continued). Specific proteins revealed at 72 h of incubation of *Trichinella pseudospiralis* (ISS7626) muscle larvae.

No.	Accession*	Proteins and groups of proteins
40-41	KRZ40966.1, KRZ40965.1	Myosin-4, Myosin-4, partial
42	KRZ39696.1	Calsyntenin-1, partial
43-46	KRZ44682.1, KRZ44684.1, KRZ44683.1, KRZ44685.1	Mesocentin
58-59	KRZ41001.1, KRZ41000.1	Disks large -like protein 1, partial
60	KRZ38095.1	Nucleoredoxin-like protein 2
61	KRZ39420.1	Neuroglian
62	KRZ32813.1	Transthyretin-like protein 46
63-67	KRZ35739.1, KRZ43176.1, KRZ45247.1, KRZ45248.1, KRZ35755.1	Uncharacterised proteins: T4C_456, partial; T4C_13990, T4C_3099, T4C_3099, T4C_13885
68	KRZ37988.1	Deoxyribonuclease-2-alpha, partial
69	KRZ38134.1	Latent-transforming growth factor beta-binding protein 2
70	KRZ25913.1	Lamin-C
71	KRZ36869.1	Sulfhydryl oxidase 1, partial
72-73	KRZ32369.1, KRZ32370.1	Laminin subunit beta-1
74	KRZ35808.1	Tropomyosin
75	KRZ45345.1	Protein disulfide-isomerase A3
76-77	KRZ39010.1, KRZ39009.1	ADP-ribose pyrophosphatase, mitochondrion
78	KRZ36799.1	Calreticulin, partial
79	KRZ46263.1	Eukaryotic translation initiation factor 5A-2
80	KRZ29422.1	L-lactate dehydrogenase
81	KRZ45561.1	Glutathione peroxidase
82	KRZ37663.1	Stress-induced-phosphoprotein 1
83-85	KRZ40062.1, KRZ40065.1, KRZ40064.1	Laminin subunit alpha-2, partial
86-88	KRZ37428.1, KRZ37429.1, KRZ37427.1	Fibropellin-1
89-90	KRZ26387.1, KRZ26386.1	Adenylosuccinate lyase, partial
91	KRZ40374.1	Glyceraldehyde-3-phosphate dehydrogenase
92	KRZ43523.1	Antileukoproteinase
93	KRZ38487.1	Adenylate kinase isoenzyme 1
94	KRZ31167.1	Phosphoglucomutase-1, partial
95	KRZ36428.1	Calreticulin
96	KRZ25817.1	Dihydrolipoyllysine-residue acetyltransferase component of pyruvate dehydrogenase complex, mitochondrial, partial
97	KRZ44865.1	Macrophage migration inhibitory factor-like protein

* – accession number according to UniProt database – *T. pseudospiralis*.

Gene ontology (GO) enrichment analysis. To find out the possible role of the proteins detected in the cultural medium in the current work we performed GO enrichment analysis.

We determined if there is any GO category enrichment in the proteins found. For that we used top.GO R package, testing enrichment in three possible ontologies: biological process (Table 6), molecular function (Table 7) and cellular component (Table 8).

Clustering and selection analysis. We determined orthologous groups (OG) for each of 69

Table 6. Enriched GO terms exceeding *P*-value of 0.01 (Biological Process). Enrichment analysis was carried out for the list of proteins detected in the medium at all incubation times (24, 48 and 72 h) of *Trichinella pseudospiralis* (ISS7626) muscle larvae.

No.	GO.ID	Term	Annotated	Significant	Expected	Adjusted *P*-value
1	GO:0006637	acyl-CoA metabolic process	3	3	0.48	0.0039
2	GO:0035383	thioester metabolic process	3	3	0.48	0.0039
3	GO:0006732	coenzyme metabolic process	12	6	1.92	0.0054
4	GO:0051186	cofactor metabolic process	12	6	1.92	0.0054
5	GO:0006753	nucleoside phosphate metabolic process	13	6	2.08	0.0088
6	GO:0009117	nucleotide metabolic process	13	6	2.08	0.0088
7	GO:0044283	small molecule biosynthetic process	13	6	2.08	0.0088

Table 7. Enriched GO terms passing *P*-value of 0.01 (Molecular Function). Enrichment analysis was carried out for the list of proteins detected at all incubation times (24, 48 and 72 h) of *Trichinella pseudospiralis* (ISS7626) muscle larvae.

No.	GO.ID	Term	Annotated	Significant	Expected	Adjusted *P*-value
1	GO:0005319	lipid transporter activity	5	5	0.81	9.6e-05
2	GO:0030234	enzyme regulator activity	11	7	1.78	0.00041
3	GO:0004866	endopeptidase inhibitor activity	6	5	0.97	0.00050
4	GO:0061135	endopeptidase regulator activity	6	5	0.97	0.00050
5	GO:0098772	molecular function regulator	16	8	2.59	0.00134
6	GO:0004857	enzyme inhibitor activity	10	6	1.62	0.00177
7	GO:0030414	peptidase inhibitor activity	10	6	1.62	0.00177
8	GO:0061134	peptidase regulator activity	10	6	1.62	0.00177
9	GO:0004867	serine-type endopeptidase inhibitor activity	5	4	0.81	0.00277
10	GO:0004518	nuclease activity	6	4	0.97	0.00729
11	GO:0004519	endonuclease activity	6	4	0.97	0.00729
12	GO:0004520	endodeoxyribonuclease activity	6	4	0.97	0.00729
13	GO:0004531	deoxyribonuclease II activity	6	4	0.97	0.00729
14	GO:0004536	deoxyribonuclease activity	6	4	0.97	0.00729
15	GO:0016889	endodeoxyribonuclease activity	6	4	0.97	0.00729
16	GO:0016894	endonuclease activity	6	4	0.97	0.00729
17	GO:0016788	hydrolase activity, acting on ester bond	13	6	2.11	0.00963

Table 8. Enriched GO terms passing *P*-value of 0.01 (Cellular Component). Enrichment analysis was carried out for the list of proteins detected at all incubation times (24, 48 and 72 h) of *Trichinella pseudospiralis* (ISS7626) muscle larvae.

No.	GO.ID	Term	Annotated	Significant	Expected	Adjusted *P*-value
1	GO:0030054	cell junction	29	21	7.1	9.7e-09
2	GO:0030056	hemidesmosome	16	13	3.92	1.3e-06
3	GO:0030055	cell-substrate junction	17	13	4.16	4.6e-06
4	GO:0005576	extracellular region	33	17	8.08	0.00032
5	GO:0005882	intermediate filament	4	4	0.98	0.00336
6	GO:0045111	intermediate filament cytoskeleton	4	4	0.98	0.00336
7	GO:0099513	polymeric cytoskeletal fiber	4	4	0.98	0.00336
8	GO:0005578	proteinaceous extracellular matrix	6	5	1.47	0.00381
9	GO:0005604	basement membrane	6	5	1.47	0.00381
10	GO:0044420	extracellular matrix component	6	5	1.47	0.00381
11	GO:0044421	extracellular region part	28	13	6.85	0.00640

E-S and somatic proteins in the PP proteins group found in all analysed samples throughout all time periods (24-72 h) (Table 9: Supplemental material).

This analysis confirmed that most of 69 PP proteins are highly species-specific: 46 out of 62 OG contain only *T. pseudospiralis* proteins. Moreover, many OG contain only proteins that are specific for few *T. pseudospiralis* strains and, therefore, can be considered strain-specific (examples: clusters 3, 5, 7, 9).

Some OG contain paralogous genes for at least one species in a group (examples: clusters 11, 23, 33, 40; total: 18 clusters), which indicate recent gene duplication events. Multiple sequence alignments of protein sequences suggest that 51% of the proteins are highly conserved within OG (identity within cluster is greater than 99%), which can also point on recent emergence of OG.

We further investigated the ratio of synonymous to non-synonymous substitutions along each codon in orthologous protein alignments. We found the signs of positive selection pressure (ω value > 1 for site, M2 model of Codeml) in 23 OG. This result indicates that relatively high portion of E-S proteins is under selective pressure, which points to the important role that these proteins play in parasite's life cycle and adaptation to a specific host.

Clustering was performed for all predicted proteins as described above; only 62 clusters that contain 69 E-S and somatic proteins produced by *T. pseudospiralis* ISS7626 and detected at all three days of incubation are shown in Table 9 (Supplemental material). The best meaningfull blast description (if any) is given for a cluster using a respective protein sequence for a cluster given by cd-hit program. 'Positive selection' column lists if a positive selection is detected by site model M2 within cluster. *Trichinella pseudospiralis*-specific clusters are the clusters that do not include proteins from other species. Highly conserved clusters include sequences with identity greater than 99%.

DISCUSSION

A detailed investigation was undertaken of proteins obtained during prolonged incubation of the zoonotic nematode *T. pseudospiralis* ISS7626 muscle larvae *in vitro*. We found that the number of revealed proteins increases with prologation of incubation, which correlates with decrease of reproduction potential and can indicate that, together with E-S proteins, accummulation of some somatic ones takes place, most probably originating from dead larvae.

Pair-wise comparisons of proteins showed that a majority of the revealed proteins emerge after 48 h of incubation and remain in the products obtained at 72 h being absent at 24 h. Among these proteins we found cell-death-related nuclease 7, transmembrane matrix receptor MUP-4, collagen alpha-6 (VI) chain, basement membrane proteoglycan, ADP-ribose pyrophosphatase mitochondrion and some others, which can be attributed to somatic proteins. At the same time in the protein repertoires at 48 h and 72 h of incubation we found 69 proteins common with those revealed at 24 h. These 69 proteins almost completely represent the ones defined for the period of 24 h, when the reproduction potential was the highest, which indirectly confirms that even after 72

h of incubation most of ML were physiologically active. They retained invasing activity, although reproduction potential decreased. Thus, we can conclude that the majority of larvae were alive during the investigated period (24-72 h) and the detected 69 PP proteins could be presented predominantly by E-S products. However, we also identified some somatic proteins among the 69 PP proteins including myoglobin, mitochondrial-processing peptidase subunit alpha, mitochondrial elongation factor Ts, poly(U)-specific endoribonuclease-like protein and some others, which means that even at short periods of incubation some larvae perish, releasing these proteins into the medium.

We can assume that the revealed 69 PP proteins including E-S and somatic ones can represent proteins in contact with the cells of the host *in vivo*. Among these proteins we have identified transmembrane protease serine 9, 5'-nucleotidase, snake venom 5'-nucleotidase and deoxyribonuclease-2-alpha, similar to those identified in E-S products of *T. pseudospiralis* KU 357408 ML (Odoevskaya *et al.*, 2018b) and referred to as immunogenic by other investigators (Bien *et al.*, 2012, 2015; Wang *et al.*, 2014), and some others revealed recently in E-S products of *T. pseudospiralis* adult worms and in somatic extracts of *T. pseudospiralis* ML: poly-cysteine and histidine-tailed protein, serine protease, chymotrypsin-like elastase family member 1, deoxyribonuclease II superfamily, GLIPR1-like protein 1, elongation factor 1-alpha (Wang *et al.*, 2017; Somboonpatarakun *et al.*, 2018). We found that 30 proteins are annotated as 'hypothetical', which usually means that computationally predicted gene models in the genome encodes proteins with no clear sequence homology to any known functionally annotated proteins. Our results thus confirm the existence of gene products at protein level and also point out that big portion of E-S proteins belong to new, unexplored protein families. The presence of certain immunogenic proteins in the group of PP proteins obtained from *T. pseudospiralis* ISS7626 ML may indicate the high virulence of this nematode.

We made an attempt to study in detail the repertoire of E-S and somatic proteins obtained during prolonged incubation of ML *in vitro* (24-72 h) as we considered that analyses of 69 PP proteins that were also identified at a short period of incubation (24 h) was not enough to follow possible alterations of the protein repertoire that may take place later. In fact among the proteins not detected at 24 h, but emerging at 48 and remaining at 72 h we identified some that can play an essential role in host-parasite coexistence: vitellogenin, that is known to increase stress resistance (Fischer *et al.*, 2013), leukocyte elastase inhibitor C, onchocystatin, a secretory antigen OV-16 antigen (Lobos *et al.*, 1991), an extracellular matrix glycoprotein Papilin (Kramerova *et al.*, 2000), antigen-like protein, an important immunoregulatory protein disulfide-isimerase 2, antioxidant protective proteins peroxiredoxin-2 and heat shock protein 83 (Wang *et al.*, 2021), which confirms the idea that proteins important for the survival of larvae *in vivo* can be traced only during prolonged incubation *in vitro*. Identification of these proteins can be essential for an effective targeted therapy of this disease and to prevent severe clinical manifestations when E-S and somatic proteins accumulate in the host muscles.

Proteins specific and unique for different time periods identified at 48 h (13 proteins) and at 72 h (97 proteins) are also of interest. In general, changes in the number of specific proteins correspond to an increase in the total number of detected proteins with time and, although it occurs together with a decrease of reproduction potential, we can suppose that appearance of these proteins may be caused not only by death and destruction of part of the larvae, but also by some changes in physiological activity of the living ones. We cannot exclude that these proteins are also interacting with the host cells *in vivo*, but we did not attempt to investigate it here as we consider it a separate task.

An essential supplement to data on the revealed total repertoire of E-S and somatic proteins and the dynamics of excretion-secretion and accumulation of these proteins is the gene ontology analysis, giving insight into their role in the larval metabolism and relationship with the host. Proteins revealed in this investigation were categorised according to standard GO domains: biological process, molecular function and cellular component. No specific enrichment can be described by the biological process ontology. Molecular function ontology shows that most of these proteins are nucleases, proteases or their regulators. Fraction of E-S proteins appears to be enriched with extracellular compartment-related proteins (GO:0005576, GO:0044420, GO:0044421) and proteins involved in cell attachment (GO:0030054, GO:0030056).

We consider these data are very important and unique, as we failed to find any references on GO analysis of total E-S proteins repertoire of *T. pseudospiralis* ML. Available data on GO analysis of *T. pseudospiralis* ML proteins were obtained with the use of somatic extracts, showing obviously different results: biological process was

characterised by oxidation-reduction process, gluconeogenesis, translational elongation, DNA metabolic process and translation, GTP binding, ATP binding, oxidoreductase activity and some others. Cellular components were associated with ribosome, integral component of membrane and cytoplasm (Somboonpatarkun *et al.*, 2018).

Excretory-secretory proteins appear at the interface between host and parasite at muscle larvae stage and can be extremely important for successful parasitism. These proteins are directly recognised by the host's immune system, which might make significant selection effect on their sequences. Our analysis reveals strong evidence of positive selection acting on various sites in more than 50% of proteins from the E-S set, which suggests that most of E-S proteins keep evolving. Interestingly, in contrast with our results, no significant enrichment with positively selected genes was found in the cestode *Echinococcus multilocularis*, the causative agent of human alveolar echinococcosis (Wang *et al.*, 2015). The possible explanation for that can be found in *T. pseudospiralis* population dynamics and natural lifecycle. More than 30 different isolates of *T. pseudospiralis* from both mammals and birds have been detected (Pozio, 2007), indicating that this worm can infect a range of different hosts. This can explain the evolutionary pressure that acts on rapidly evolving and possibly recently emerged protein groups. Our orthologous clustering can also support this idea, showing that most E-S proteins are *T. pseudospiralis*-specific or even strain specific. High level of sequence identity within clusters and frequent presence of paralogous genes within cluster are in good agreement with the idea of recent emergence of these OG. We can hypothesise that the set of E-S proteins of *T. pseudospiralis* contains recently emerged and rapidly evolving proteins, which may help this parasite to adapt to a wide range of hosts.

CONCLUSIONS

The analysis of the total repertoire of E-S and somatic proteins accumulated during incubation of ML *in vitro* convinces us that these proteins can be considered as those directly participating in the parasite-host relationships *in vivo*. This repertoire can serve as a basis for further investigation and selection of proteins most crucial for the long existence of host-parasite complex and promising for development of targeted therapy of the desease. We can also hypothesise that the set of 69 PP proteins revealed during prolonged incubation of *T. pseudospiralis* larvae contains recently emerged and rapidly evolving proteins, which may help this parasite to adapt to wide range of hosts.

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SUPPLEMENTAL MATERIAL

Table 9. Amino acid sequence-based clustering of *Trichinella* proteins. Clustering was performed for all predicted proteins as described in Materials and methods; only 62 clusters that contain 69 proteins of *T. pseudospiralis* ISS7626 detected at all three days of cultivation are shown in the Table. The best meaningful blast description (if any) is given for a cluster using a respective protein sequence for a cluster given by cd-hit program. 'PS' column indicates a positive selection is detected (site model, M2) within a cluster. 'SS' column indicates *T. pseudospiralis*-specific clusters that are the clusters that do not include proteins from other species. 'HC' column indicates highly conserved clusters that include sequences with identity greater than 99% only. – http://www.russjnematology.com/Articles/rjn302/Paper3_Odoevskaya_Table9_SUPPL.pdf

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И.М. Одоевская, Е.С. Герасимов, И.Б. Кудряшова, Л.И. Качурина, В.В. Рекстина, Р.Х. Зиганшин и Т.С. Калебина. Анализ репертуара белков, обнаруженных в среде при длительной инкубации *in vitro* мышечных личинок *Trichinella pseudospiralis*.

Резюме. Белки, накапливающиеся в среде при длительной инкубации мышечных личинок *Trichinella pseudospiralis* ISS7626 (домашняя свинья *Sus scrofa* (Камчатка)) анализировали с помощью LC-MS/MS. Количество выявленных экскреторно-секреторных и соматических белков возрастает с 72 до 238 по мере инкубации, пропорционально уменьшению потенциала воспроизводства личинок, остающихся при этом инвазивными. Анализ представленности функциональных групп генов (по базе Gene Ontology) показал, что большая часть выявленных белков – нуклеазы, протеазы или их регуляторы; превалируют также белки, связанные с внеклеточным компартментом, и белки, участвующие в прикреплении клеток. 69 из выявленных белков постоянно присутствуют во всех проанализированных образцах, что коррелирует с подтверждённой в биопробе на белых мышах жизнеспособностью личинок на протяжении всего периода инкубации. Анализ ортологичных групп генов показал, что большая часть постоянно присутствующих белков является видоспецифичной для *T. pseudospiralis*. Высокая степень сходства последовательностей и частое присутствие паралогичных генов внутри ортогруппы может указывать на недавнее происхождение и быстрое эволюционирование этих белков в процессе адаптации к широкому диапазону хозяев.