Metoder för strategisk kontroll och övervakning av resistens hos *Fasciola hepatica* i Sverige (H1350023)

Strategic control program against fasciolosis and monitoring of resistance against *Fasciola hepatica* in Sweden (H1350023)

Report author(s)

Huvudsökande:

- Johan Höglund - Institutionen för biomedicin och veterinär folkhälsovetenskap, Sektionen för parasitologi (BVF), SLU, Uppsala

Medsökande:

- Adam Novobilský - Institutionen för biomedicin och veterinär folkhälsovetenskap, Sektionen för parasitologi (BVF), SLU, Uppsala
- Katarina Gustafsson - Gård & Djurhälsan (G&D), Länghem

Background

Fasciola hepatica is a trematode parasite with a world-wide distribution, which is responsible for considerable disease and production losses in ruminants. Infections with *F. hepatica* (fasciolosis) in cattle and sheep can affect growth, carcass composition and fertility. Recent reports have shown increasing prevalence of fasciolosis in Europe. Control of fasciolosis is based strictly on annual deworming of animals using flukicides. Recently, *F. hepatica* resistant isolates to common flukicides, such as triclabendazole (TCBZ) and albendazole (ABZ), have been reported in UK, The Netherlands, Australia and Argentina. In Sweden, albendazole has been used as the only flukicide for more than 40 years. Recently, albendazole failure has been recorded in sheep naturally infected with *F. hepatica* in south-west Sweden. Reasons of the failure, however, remained unclear. Thus, control of fasciolosis and drug efficacy against live flukes in Sweden requires more attention.

The main aims of the project were to; (1) develop suitable method for detection of anthelmintic resistance against *F. hepatica* in Swedish livestock; (2) investigate effects of ABZ and TCBZ against liver flukes in ewes when dewormed in different time periods; and (2) evaluate efficacy of closantel treatment in beef cattle;.

Materials and methods

Development of diagnostic methods

Several methods were tested both in the field and in laboratory to develop reliable diagnosis of flukicide efficacy. Effects of tested drugs against liver flukes in animals were measured by faecal egg count reduction test (FECRT), coproantigen reduction test (CRT) in principle according previous studies. Furthermore, an *in vitro* Fasciola egg hatch test (FEHT) for testing susceptibility of *Fasciola* eggs to albendazole was conducted. In FEHT, fluke eggs were exposed to ABZ at a final concentration of 0.02, 0.1, 0.5, 2.5, 12.5 nmol/ml and 0.5% dimethylsulfoxid (DMSO) only (negative control) for 12 h at 25°C. Each concentration was tested in four replicates containing approximately 100 eggs per well. After exposure, the eggs were washed three times in tap water and then incubated in 24-well plastic plates in 2 ml tap water for 14 days at 25°C in the dark. After incubation, miracidial hatching was stimulated by exposure to intensive light for 12 h. Hatching was terminated by adding of 0.1 ml Lugol's iodine solution to each well 4 h after exposure to light. Hatched and unhatched, including dead eggs and eggs with developed miracidia were counted under an inverted microscope at 40x magnification. Ovicidal activity of ABZ was calculated according to the formula, below: Ovicidal activity (%) = (% of eggs hatched in negative control - % eggs hatched after drug

exposure/% of eggs hatched in negative control)*100. FEHT was optimized and tested using 8 different Swedish *F. hepatica* isolates of both bovine and sheep origin. FEHT was also tested with closantel (CLS) and triclabendazole (TCBZ).

Molecular methods for diagnosis of *F. hepatica* in animals and diagnosis of flukicide resistance were developed. Polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) were carried out using newly designed primers. Specificity and sensitivity of both methods were tested using DNA obtained from various helminth species. PCR and LAMP were then tested using sheep and cattle faecal samples collected in naturally infected animals and compared to conventional methods such as serology, faecal egg counts and coproantigen ELISA.

Usefulness of immunodiagnostic methods in screening and surveillance of fasciolosis at country and national level was evaluated. Individual serum samples from 2135 beef cattle herds and bulk-tank milk samples from 435 dairy herds were examined for the presence of specific anti-*Fasciola hepatica* antibodies using in-house ELISA and/or Svanovir ELISA kit (SVANOVA).

Field work

Drug trial in cattle

The efficacy of closantel (CLS) against *F. hepatica* was studied in 3 selected beef cattle herds in Västra Götaland (Horred, Gröna Gårdar, and Henån) between January and March 2014. One week before application, 25 % of cows were sampled and examined for faecal egg counts (FEC) (sedimentation method) and coproantigen (coproantigen ELISA method) on each farm. 10 animals that were both egg and coproantigen positive were selected for further screening of drug efficacy. At day 0, all animals were dewormed with recommended dose of CLS (commercial product Closamectin pour on containing closantel and ivermectin). Samples were collected from selected cows at days 0, 7, 21 days after application and were examined both by sedimentation and coproantigen ELISA.

Drug trial in sheep

Between November 2014 and January 2015, two deworming trials were performed on two sheep farms (farms A and B) in south-west Sweden. On each farm, *F. hepatica* positive animals were selected in initial prescreening using coproantigen ELISA (cELISA). *Fasciola hepatica* positive ewes were then randomly divided into groups (n=6-8) and treated with ABZ or TCBZ in January 2015 according to recommended dose. Animals were sampled at days 0, 7 and 21 days after drug application. To compare the efficacy of ABZ between autumn and winter deworming, other two groups of ewes were treated with ABZ or TCBZ on farm A in November (4 weeks after housing). Drug efficacy was evaluated using FECRT and CRT. Irrespective of drug trial, liver fluke egg isolates were collected from both flocks before the field experiment. Eggs of both isolates were exposed to various concentrations of albendazole and hatching rate after exposure was observed (FEHT).

Results

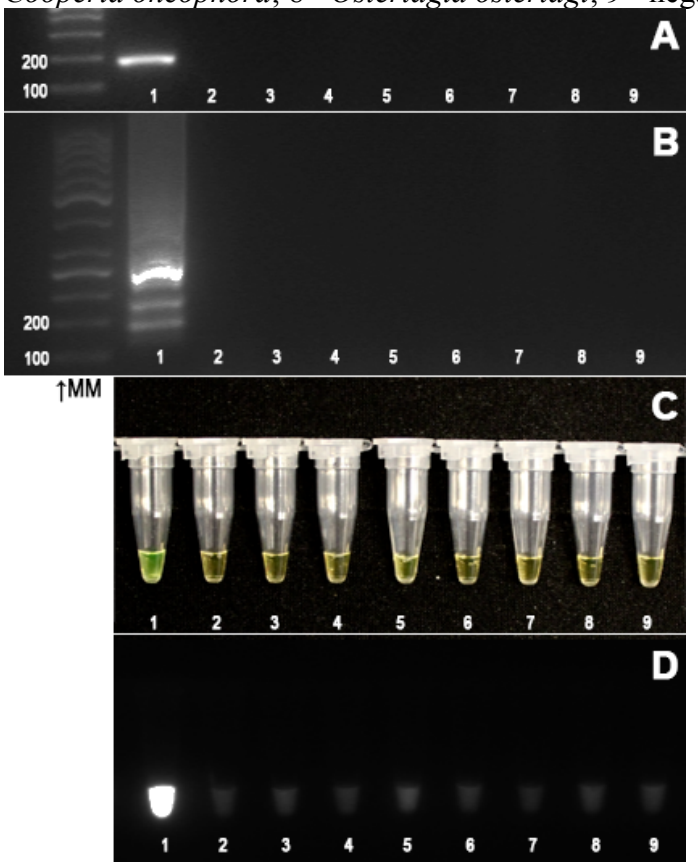
Development of diagnostic methods

During 2014, the FEHT was optimized in our laboratory using several *F. hepatica* isolates. Exposure time for eggs has been set up to 12 hours. We also tested different concentrations of dimethylsulfoxid (DMSO) which is used as dissolvent for the drug. Maximum concentration that does not affect larval development of eggs after 12-hour exposure was 0.5-1% DMSO. Various doses of ABZ, TCBZ and CLS were tested with different *F. hepatica* isolates.

Discriminating dose for sensitivity of *F. hepatica* eggs to ABZ was established between 0.5 and 1.5 nmol/ml. For TCBZ and CLS, the protocol of the FEHT needs to be modified due to problems of solubility of those drugs in DMSO. For more details see **Novobilský et al. 2016**.

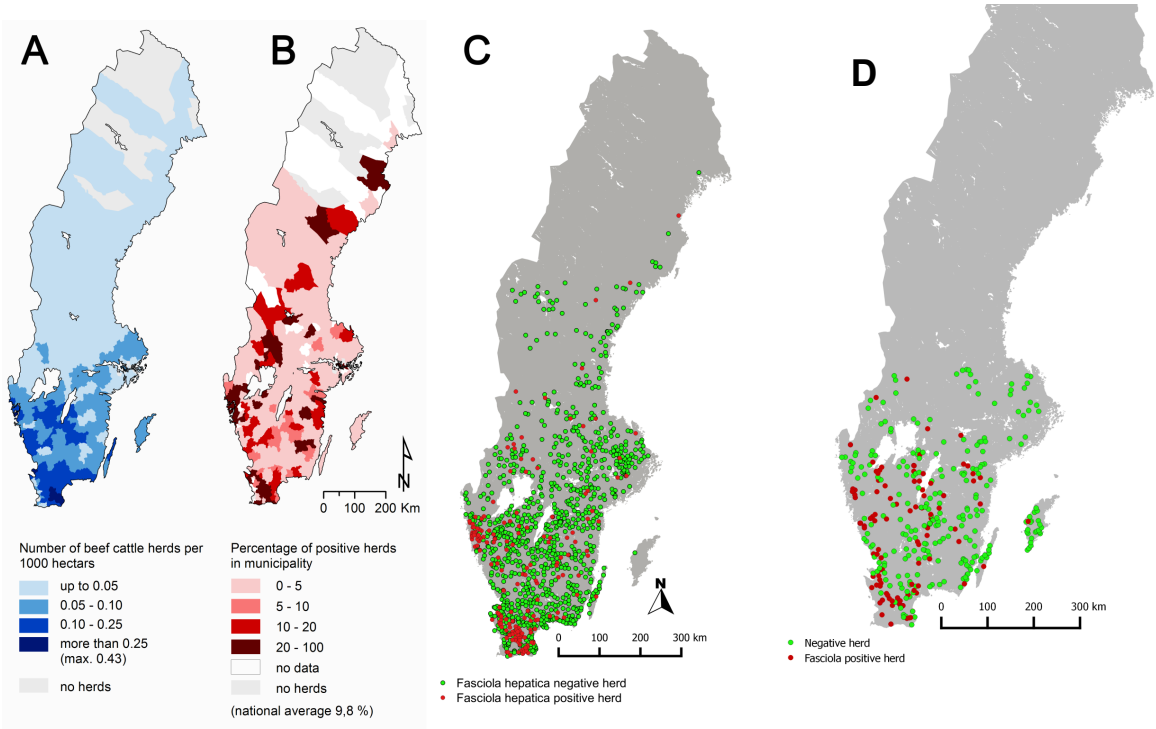
When tested with DNA isolated from adult parasites, both PCR and LAMP were highly sensitive and specific (**Figure 1**). A total of 64 sheep and beef cattle samples were collected on 4 farms in south-west Sweden. Of 64 animals examined, 53 were positive for anti-*F. hepatica* antibodies, 36 were positive by cELISA and 28 were egg count positive. EPG values positively correlated with cELISA levels ($R=0.81$, $p<0.001$). Significant positive correlations were also found between cELISA and serology ($R=0.66$, $p<0.001$) and between FEC and serology ($R=0.55$, $p<0.001$). Only 3 and 6 animals, respectively, were detected positive for *F. hepatica* by PCR and LAMP. Compared with FEC and cELISA data, sensitivity and specificity of PCR was 10.7% and 100%, respectively. Sensitivity and specificity of LAMP method was 17.9% and 97.2%, respectively (see **Arifin et al. 2016**).

Figure 1. Specificity of PCR (A) and LAMP methods (B, C, D) using DNA of various trematode and nematode species. A: gel electrophoresis of PCR products; B: gel electrophoresis of LAMP products; C: colorimetric visualisation of LAMP products with SYBR Green in reaction tubes; D: LAMP reaction tubes under UV light. Annotations: MM-molecular marker, 1 - *Fasciola hepatica*, 2 - *Dicrocoelium dendriticum*, 3 - *Paramphistomum cervi*, 4 - *Calicophoron daubneyi*, 5 - *Haplometra cylindracea*, 6 - *Haemonchus contortus*, 7 – *Cooperia oncophora*; 8 - *Ostertagia ostertagi*; 9 - negative control.



Overall herd seroprevalence of *F. hepatica* in beef cattle was 10%. An irregular spatial distribution of *F. hepatica*, with two main clusters, was observed in south-west Sweden **(Figure 2).** In dairy cattle, seroprevalence was 25% and the main risk factor was the length of grazing period for heifers. See **Novobilský et al. 2015 a, b**.

Figure 2. Spatial distribution of *F. hepatica* in beef (A, B, C) and dairy cattle (D) in Sweden

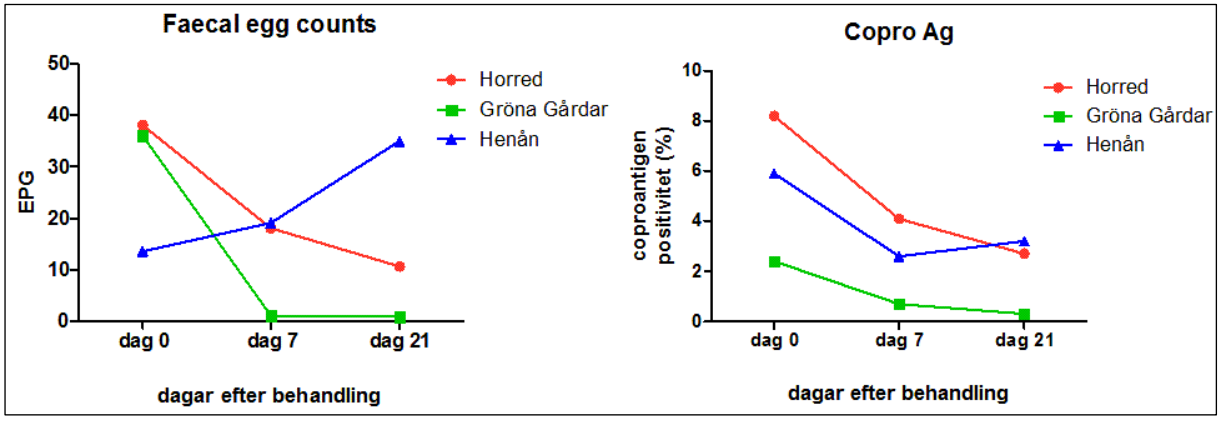


Drug trial in cattle

Based on FEC, efficacy of closantel was 72% and 97% on farms Horred and Gröna Gårdar, respectively. No reduction of *F. hepatica* egg counts at all was observed on farm Henån. Totally 4, 1 and 6 animals remained coproantigen positive 21 days after treatment on farms Horred, Gröna Gårdar and Henån, respectively. Detailed results are shown in **Figure 3**.

CLS treatment failure was confirmed on two of the farms. As the animals were housed 12-16 weeks before treatment and thereafter during the entire study, failure due to the presence of juvenile flukes was excluded. Although the cause of CLS failure currently remains unclear, development of resistance or/and absorption failure of topical administration should be considered. The detailed reasons of CLS failure are discussed in the scientific paper (Novobilský and Höglund, 2015).

Figure 3. The course of faecal egg counts and coproantigen levels (average of 10 animals) after closantel treatment on the three beef cattle farms in Västra Götaland.



Drug trial in sheep

As documented by FECRT and CRT at day 21 after deworming, both ABZ and TCBZ were highly effective (96-100% efficacy) against liver flukes on farm A. In addition, no significant difference in FEC and coproantigen (P>0.05) was observed between November and January treatment for the TCBZ and ABZ groups on farm A. On farm B, both coproantigen and FEC data showed low efficacy in the ABZ group, where 50% of animals remained coproantigen-positive 21 days post-treatment (**Table 1**). The FEHT data showed an obvious difference in sensitivity to ABZ between *F. hepatica* eggs isolated from animals on farms A and B. At a concentration of 2.5 nmol/ml, the ovicidal activity of ABZ was 100% for the ovine isolate from farm A but only 54% for the farm B isolate (**Figure 4**). The EC_{50} values for the ovine isolate from farms B was 10-fold higher than that obtained for the ovine isolate from farm A (**Table 2**). See **Novobilský et al. 2016**.

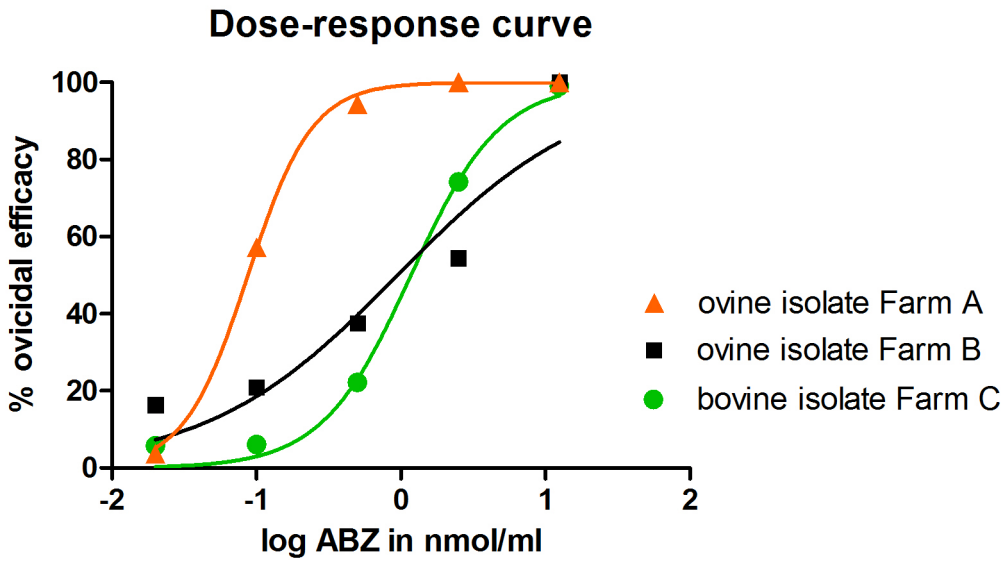
Table 1. *Efficacy of albendazole and triclabendazole against liver flukes based on faecal egg counts (FEC) and coproantigen ELISA tests on faecal samples obtained 7 and 21 days post-treatment on sheep farms on farm A and farm B*

		Sheep Farm A				**Sheep Farm B**	
		November		**January**		**January**	
		Day 7	Day 21	Day 7	Day 21	Day 7	Day 21
ABZ	Faecal egg count reduction in %	99	92	56	99	71	67
	Coproantigen reduction in %	97	97	95	98	76	32
TCBZ	Faecal egg count reduction in %	82	100	85	99	100	100
	Coproantigen reduction in %	99	98	100	99	99	97

Table 2. *Ovicidal efficacy determined by in vitro Fasciola egg hatch test (FEHT) of albendazole (ABZ) at concentrations 0.02, 0.1, 0.5, 2.5, and 12.5 nmol/ml against F. hepatica eggs of two ovine isolates (farm A and B) and one bovine isolate (farm C).*

	Ovicidal efficacy		
ABZ (nmol/ml)	**Ovine isolate (Farm A)**	**Ovine isolate (Farm B)**	**Bovine isolate (Farm C)**
12.5	100	100	100
2.5	100	54.5	74.2
0.5	94.4	37.5	22.2
0.1	57.2	20.9	6.1
0.02	3.6	16.4	5.8
EC_{50}	0.087	0.947	1.171
95% CI of EC_{50}	0.078 to 0.098	0.207 to 4.334	0.822 to 1.667
R^2	0.998	0.901	0.993

Figure 4. *Dose-response curves of albendazole (ABZ) ovicidal activity for ovine (farms A and B) and bovine (farm C) Fasciola hepatica egg isolates after probit transformation of ABZ concentrations.*



Discussion

Usefulness of serology was shown in estimating prevalence and distribution of fasciolosis at national level. Serological data combined with geographical tools such as geographical information system (GIS) can thus provide very detailed picture of exact spatial distribution of liver flukes in Sweden. In addition, GIS and seroprevalence data can be used in modelling of future disease scenarios. Since all previous seroprevalence studies have been carried out in dairy cattle in Europe, there are no available data for beef cattle. In this respect, our study in beef cattle is, therefore, unique and also shows that immunodiagnostic methods are still essential in veterinary epidemiology. The overall *F. hepatica* seroprevalence in dairy cattle in Sweden found in this study (25%) is similar to that reported for several other European countries, e.g. Belgium 37% (Bennema et al., 2009), Portugal 11-42% (Conceicao et al., 2004), Germany 24% (Kuerpick et al., 2013) and Spain 20% (Sanchez-Andrade et al., 2002). In contrast, it is much higher than that reported in a previous Swedish study in 2008, when only 7% of 205 dairy herds were infected (Höglund et al., 2010). Since prevalence in beef cattle (10%) was established on sera collected in 2006/2007, we conclude that prevalence of *F. hepatica* significantly increased during last ten years. This is also supported by meat inspection data where an almost fourfold increase was shown in *F. hepatica* prevalence in all slaughtered cattle (dairy and beef), from 3% in 2005 to 11% in 2013. Furthermore, it is evident that meat inspection data are underestimated when compared to our seroprevalence data.

Molecular diagnosis of parasitic diseases is rapidly developing and progressive topic in veterinary medicine. Our hypothesis was that parasite DNA can be detected in animal faeces using polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). When parasites are eliminated in liver by drug, parasite's cells, including its DNA, should also disappear in faeces. PCR and LAMP method were optimized in our lab and both methods showed high specificity and sensitivity when tested with purified DNA isolated form adult parasites. However, these molecular methods failed when tested with faecal samples from naturally infected animals. Low sensitivity of PCR and LAMP is most likely associated with

low concentrations of parasite DNA in faeces. Our results are contrary to previous study by Martínez-Valladares and Rojo-Vázquez (2016) where LAMP had better sensitivity than faecal egg counts and coproantigen levels. Improvement and development of the faecal DNA extraction method is needed to obtain reliable, robust and validated tests for diagnosis of *F. hepatica* in ruminants.

Diagnosis of *F. hepatica* in animals differs from diagnosis of drug efficacy/resistance. Distinguishing an animal with patent infection from a treated animal is crucial in drug efficacy/resistance diagnosis. The faecal egg count reduction test (FECRT) and coproantigen reduction test (CRT) have been suggested as suitable methods for drug efficacy/resistance diagnosis (Brockwell et al., 2013). In our project, we observed a compatibility of both methods with high level of correlation between faecal egg counts and coproantigen levels. However, for final decision about drug efficacy/resistance additional independent test is needed. Irrespective of drug trial, our novel in vitro test (FEHT) provides information about susceptibility of *F. hepatica* eggs to albendazole. Thus, the method can be used as a useful tool for identification of albendazole resistance in the field as was shown in drug trial in sheep (Novobilský et al., 2016). In addition, ABZ resistance documented in one sheep flock is the first reported case in northern Europe. Recently, ABZ has been reported from Argentina and Spain (Canevari et al., 2014; Robles-Perez et al., 2014). Finally, it needs to emphasized that FEHT can be used for diagnosis of susceptibility/resistance to ABZ only. It seems that the use FEHT is most likely limited for CLS and TCBZ due to lack of ovicidal activity against *F. hepatica* eggs.

Closantel in commercial product Closamectin was launched to Swedish market in 2011. Closamectin is currently a key anthelmintic drug against liver flukes for beef cattle in Sweden. Closantel failure reported in this project is, thus, serious finding that requires attention of beef producers, veterinarians and pharmaceutical companies. Although the cause of closantel failure currently remains unclear, development of resistance or/and absorption failure of topical administration should be considered. To our knowledge, this is the first report of closantel treatment failure against *F. hepatica* in cattle (Novobilský and Höglund, 2015).

Conclusions

Increasing prevalence of fasciolosis in Sweden requires attention regarding effective control of disease in ruminants. We showed that FECRT, CRT and FEHT can be utilized in diagnosis of drug efficacy/resistance to flukicides in Swedish livestock. Reports of ABZ and closantel failure suggest that control programs of fasciolosis needs to be revised and discussed. Annual mass deworming of sheep and beef cattle should be coordinated by veterinarians and diagnostic labs to improve efficacy of treatment and avoid selection for anthelmintic resistance in Swedish livestock.

References

Bennema SC, Vercruysse J, Claerebout E, Schnieder T, Strube K, Ducheyne E, Hendrickx G, Charlier J, 2009. Vet. Parasitol. 165, 51-57.

Brockwell, Y.M., Spithill, T.W., Anderson, G.R., Grillo, V., Sangster, N.C., 2013. Vet. Parasitol. 196, 417–426.

Canevari, J., Ceballos, L., Sanabria, R., Romero, J., Olaechea, F., Ortiz, P., Cabrera, M., Gayo, V., Fairweather, I., Lanusse, C., Alvarez, L., 2014. J. Helminthol. 88, 286–292.

Conceiçao MAP, Durao RMB, Costa IMH, Castro A, Louza AC, Costa JC, 2004. Vet. Parasitol. 123, 93-103.

Höglund J, Dahlström F, Engström A, Hessle A, Jakubek EB, Schnieder T, Strube C, Sollenberg S, 2010. Vet. Parasitol. 171, 293-299.
Kuerpick B, Conraths FJ, Staubach C, Frohlich A, Schnieder T, Strube C, 2013. Parasitololgy 140, 1051-1060.
Martínez-Valladares, M., Rojo-Vázquez, F.A., 2016. Parasit. Vectors 9:73.
Novobilský, A., Höglund, J., 2015. Int. J. Parasitol.: Drugs Drug Resist. 5, 172–177.
Robles-Perez, D., Martinez-Perez, J.M., Rojo-Vazquez, F.A., Martinez-Valladares, M., 2014. Vet. Parasitol. 203, 217–221.
Sanchez-Andrade R, Paz-Silva A, Suárez JL, Panadero R, Pedreira J, López C, Díez-Baños P, Morrondo P, 2002. Vet. Res. Commun. 26, 361-370.

Dissemination of results to the agricultural/horticultural sector

Results from the project were presented to stakeholders, including farmers, veterinarians, and scientists (see Publications: Popular science, Conference Abstracts). Farmers were informed about liver flukes and their control on specific seminars and congress organized by Gård och Djurhälsan (i.e. Nötköttsseminarium, Skövde, 2017). We communicated our results to veterinary community at Veterinary Congress, Uppsala 2016. Results were further distributed at several scientific conferences (6th Congress of Scandinavian-Baltic Society for Parasitology, International Conference of the World Association for the Advancement of Veterinary Parasitology, Liverpool, United Kingdom, 2015). Liver flukes in Sweden and control of fasciolosis were also shown to broader public on agricultural fair (ELMIA Lantbruk, Jönköping, 2016) and our website (http://adamnovobilky.wixsite.com/leverflundror/om-projekten).
As outcome of the project, a total of 5 scientific papers were published in peer-reviewed journals (see Publications: Peer-reviewed journals). In addition, two master students (Maria Arifin and Natalia Amaya Solis) were involved in the project work.

Publications

Peer-reviewed journals:

- Arifin M. I., Höglund J., Novobilský A., 2016. Comparison of molecular and conventional methods for the diagnosis of *Fasciola hepatica* infection in the field. Veterinary Parasitology 232, 8–11. http://www.sciencedirect.com/science/article/pii/S0304401716304356
- Novobilský A., Amaya Solis N., Skarin M., Höglund J., 2016. Assessment of flukicide efficacy against *Fasciola hepatica* in sheep in Sweden in the absence of a standardised test. International Journal for Parasitology: Drugs and Drug Resistance, 6 (3), 141–147. http://www.sciencedirect.com/science/article/pii/S2211320716300288
- Novobilský A., Höglund J., 2015. First report of closantel treatment failure against *Fasciola hepatica* in cattle. International Journal for Parasitology: Drugs and Drug Resistance 5 (3), 172–177. http://www.sciencedirect.com/science/article/pii/S2211320715300075
- Novobilský A., Novák J., Björkman C., Höglund J., 2015a. Impact of meteorological and environmental factors on the spatial distribution of *Fasciola hepatica* in beef cattle herds in Sweden. BMC Veterinary Research 11, 128. http://www.biomedcentral.com/1746-6148/11/128
- Novobilský A., Sollenberg S., Höglund J., 2015b. Distribution of *Fasciola hepatica* in Swedish dairy cattle and associations to pasture management factors. Geospatial Health 9 (2), 293–300. http://www.geospatialhealth.net/index.php/gh/article/view/351

Popular science:

- Novobilský A., 2016. Stora leverflundran hos svenska husdjur: En skadlig leverparasit hos nötkreatur och får. ELMIA Lantbruk, Jönköping, Oktober 19-21, 2016.
- Höglund J., Novobilský A., Gustafsson K., 2014. Stora leverflundra hos snäckor, får och nötkreatur. Vallkonferensen 2014, Uppsala, Sweden, February 5-6, 2014, pp. 67-70.
- Gustafsson, K., 2014. Vårtecken – Träckprov före betessläpp. Djurhälsonytt,1.
- Gustafsson, K. 2014. Träckprov före betessläpp. Fårskötsel, 3.
- Höglund, J, Novobilský, A, Gustafsson, K. 2014. Forskning om stora leverflundran hos snäckor, får och nötkreatur. Djurhälsonytt, 3, 16-17.

Conference abstracts:

- Novobilský A., 2017. Diagnosis and prevention of liver flukes (Fasciola hepatica) in cattle. Gård & Djurhälsans Nötköttsseminarium 2017, Skövde, January 19-20th, 2017.
- Novobilský A., Arifin M.I., Skarin M., Höglund J., 2016. Control of fasciolosis in Swedish livestock: Do we have to worry about anthelmintic resistance? Proceedings of the Veterinary Congress in Uppsala, 10-12 November, 2016.
- Novobilský A., Björkman C., Novák J., Höglund J. 2015. Spatial distribution of *Fasciola hepatica* in Swedish beef cattle herds and assessment of risk factors. Proceedings of International Conference of the World Association for the Advancement of Veterinary Parasitology, Liverpool, United Kingdom, August 16-20, 2015.
- Novobilský A., Höglund J. 2015. Failure of closantel treatment against *Fasciola hepatica* infection in beef cattle. Proceedings of International Conference of the World Association for the Advancement of Veterinary Parasitology, Liverpool, United Kingdom, August 16-20, 2015.
- Novobilský A., Björkman C., Novák J., Höglund J. 2015. Spatial distribution of *Fasciola hepatica* in Swedish beef cattle herds and assessment of risk factors. Proceedings of 6th Conference of the Scandinavian Baltic Society for Parasitology, Uppsala, Sweden, April 23-24, 2015.

Meetings and seminars with farmers:

- Gustafsson, K., 2015. Seminars organized by G&D and Länsstyrelsen: 2015-01-29 Uddevalla; 2015-02-04 Skara; 2015-02-05 Länghem