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Review Article

The potential for vaccines against scour worms of small ruminants

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ABSTRACT

This review addresses the research landscape regarding vaccines against scour worms, particularly *Trichostrongylus* spp. and *Teladorsagia circumcincta*. The inability of past research to deliver scour-worm vaccines with reliable and reproducible efficacy has been due in part to gaps in knowledge concerning: (i) host-parasite interactions leading to development of type-2 immunity, (ii) definition of an optimal suite of parasite antigens, and (iii) rational formulation and administration to induce protective immunity against gastrointestinal nematodes (GIN) at the site of infestation. Recent 'omics' developments enable more systematic analyses. GIN genomes are reaching completion, facilitating "reverse vaccinology" approaches that have been used successfully for the *Rhipicephalus australis* vaccine for cattle tick, while methods for gene silencing and editing in GIN enable identification and validation of potential vaccine antigens. We envisage that any efficacious scour worm vaccine(s) would be adopted similarly to "Barbervax™" within integrated parasite management schemes. Vaccines would therefore effectively parallel the use of resistant animals, and reduce the frequency of drenching and pasture contamination. These aspects of integration, efficacy and operation require updated models and validation in the field. The conclusion of this review outlines an approach to facilitate an integrated research program.

1. Introduction

Gastrointestinal nematodes (GINs) cost the Australian sheep and goat industries approximately AUD 450 million per annum (Lane et al., 2015), amounting to approximately 8% of the value of the Industry. In Australia, and internationally, a significant proportion of this cost is attributable to *Haemonchus contortus* ("Barber's pole worm") and the combined impact of two other genera of "scour worms", including *Teladorsagia circumcincta* ("small brown stomach worm") and *Trichostrongylus axei* ("stomach hair worm") and small intestinal GINs, *Trichostrongylus colubriformis* and *Trichostrongylus vitrinus* ("black scour worms"). Several other scour worms, including *Nematodirus* spp. ("Thin-necked intestinal worm"), *Cooperia* spp. ("intestinal hair worms"), and the large intestinal nematodes *Chabertia ovina* ("large-mouth bowel worm") and *Oesophagostomum* spp. ("large bowel worms") add to the production losses. Mixed infections are the norm, the proportional compositions varying between countries, regions and enterprises.

Successful integrated parasite management programs (IPMs) (Kahn and Woodgate, 2012) involve the "prevention-detection-response" approach where strategic use of anthelmintic treatments is key alongside pasture management, breeding programs for natural resistance or resilience, nutritional management, effective diagnosis and drench efficacy testing. With growing concerns over the re-emergence of widespread anthelmintic resistance in *Tel. circumcincta* and *T. colubriformis,* and more erratic seasonal weather, refugia-based strategies (e.g. targeted drenching) have been introduced to IPM programs to prolong drench efficacy. Vaccines offer an optional and sustainable component of IPM programs.

Vaccines have successfully controlled infectious diseases, utilising the host's protective immune responsiveness to limit pathology and production loss. The recent success of Barbervax™, which achieves approximately 80% protection against *H. contortus* through induction of high titres of antibody by repeated inocula of a native gut antigen preparation (Le Jambre et al., 2008; http://barbervax.com.au/), raises the possibility for vaccination against other GINs. This may require the induction of an effective mucosal immune response in these species which do not ingest blood. With technological expansion in the development, design

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and delivery of vaccines, a re-appraisal of this potential is timely. It is likely that specific vaccines will need to be developed to control each species, although a "pan-species" vaccine would be the ideal product. To this end, this review has three aims:

(i) to review literature on scour worm vaccines to provide reasons for the lack of (reproducible) success;
(ii) to examine new technologies and advances in the 'omics' areas which enable more incisive research into host-parasite interaction, antigen identification, vaccine formulation and delivery
(iii) to provide an integrated approach for the use of vaccines in IPMs

2. Development of immunity to scour worms

2.1. Cross protection against GINs

Since most GINs infestations are mixed, many reports have identified physiological and immunological regulation of parasites in preferred niches in the GI tract in small ruminants with single (bolus) or continuous (trickle) doses of infective L3s (iL3). Amongst three abomasal GINs, combined infections reduced *H. contortus* by 50–90%, while *Tel. circumcincta* and *T. axei* were unaffected (Dobson and Barnes, 1995). Gruner et al. (2002) studied populations of *Tel. circumcincta* and *T. colubriformis* in resistant (R) and susceptible (S) rams, demonstrating that R ram lambs effected a 25% and 96% reduction in pasture L3 populations of *Tel. circumcincta* and *T. colubriformis*, respectively, after 2 years, consistent with more effective immunity against *T. colubriformis*.

2.2. Host-parasite interactions related to immunity

2.2.1. Induction and expression of immunity to scour worms

"Resistance" to GINs is synonymous with the establishment of innate and acquired immune responses. Resistance has a moderate heritability (h^2; see below), enabling selection for reduced faecal worm egg counts (FWEC). Immunity is commonly assessed by reductions in FWECs, accompanied by larval differentiations (or PCR) from faeces if the proportions of species are required; recent development of nemabiome sequencing (metabarcoding) may supercede this (Avramenko et al., 2015). When necropsies are performed, immunity can also be expressed as reductions in total and differential worm counts, worm length and the number of eggs in the uterus.

"Resilience" is usually defined as the ability of small ruminants to thrive during field challenge with GINs. Resilience has a small genetic component ($h^2 \sim 0.1$) and is measured by liveweight gain, fleece or meat production characteristics, and/or increased drenching intervals (Bisset et al., 1996, 2001). The mechanism of resilience is incompletely understood and resilient animals perform less well under nutritional stress (MLA, 2017).

Development and expression of "conventional" immunity. In naïve hosts, establishment rates for GIN range from 40% to 80% (Dobson et al., 1990; Gaba et al, 2006). Initial losses of GIN are caused by innate inflammatory responses, as establishment is enhanced by pre-treatment with corticosteroids (Emery, 1996; Miller, 1996). With continuous ingestion of iL3s, small ruminants develop an age-related resistance to worm infestations (Fig. 1). The interval of 4–9 weeks for the expression of immunity to *T. colubriformis* L3s (Dobson et al., 1990) was similar to that observed in lambs trickle infected with *H. contortus* L3s (Barger et al., 1985). The seasonal ingestion of new cohorts of *H. contortus* L3s in Spring elicited a "self-cure" reaction (Stewart, 1955; Blitz and Gibbs, 1972; Adams, 1993) and eliminated other abomasal and intestinal GINs (Stewart, 1955; Emery et al., 1993; Harrison et al., 1999). Sheep immune to GIN that have been exposed within the previous 7 weeks can mount a 'rapid rejection' response that prevents establishment of *H. contortus* L3s within 30 min (Miller, 1996) and eliminates incoming *T. colubriformis* L3s from the entire 15 m of the small intestine within 2 h (Wagland et al., 1996). While optimal immunity removed GIN entirely (Wagland et al., 1996), lesser levels of immunity resulted in establishment further downstream in the small intestine (Harrison et al., 1999), indicating the reversible paralysis of motility by the "mucosal effector response" (Emery et al., 1993; Harrison et al., 2008). The kinetics were accelerated in genetically resistant sheep (Windon, 1996). The immune system of

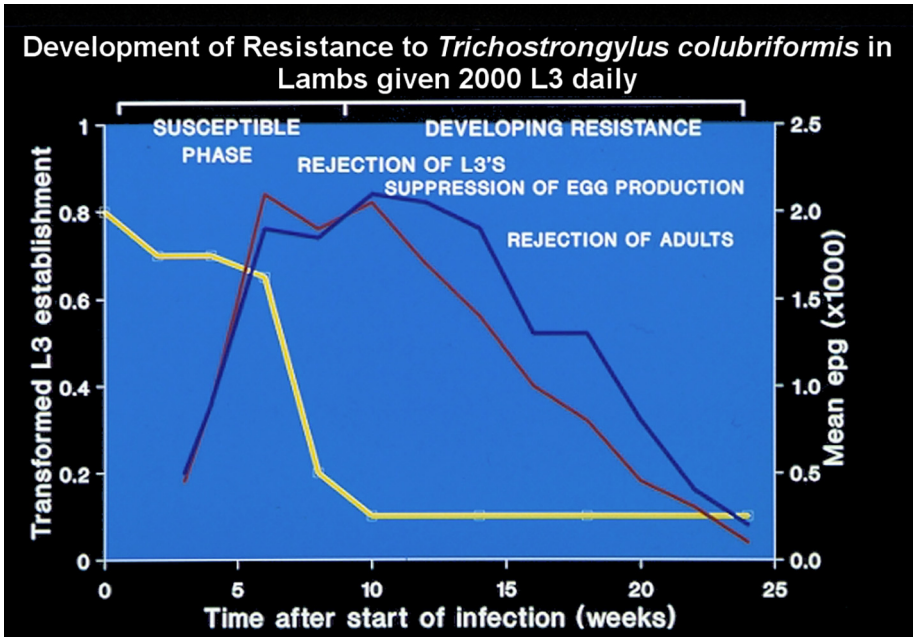

Fig. 1. Kinetics of the development of escalating immunity after infection of lambs dosed with 2000 *Trichostrongylus colubriformis* L3s per day (adapted from Dobson et al., 1990). Comparisons include: % L3 establishment (yellow (light grey)), egg production (as faecal worm egg count, red (medium grey)) and worm counts (blue (dark grey)).

"resistant" animals may react more effectively to lower amounts of worm antigen to limit infections or reject worms. They may remove worms with less collateral damage in the gut, or forage better for dietary protein, which is essential for optimal development of immunity to worms (Coop et al., 1995; van Houtert et al., 1995).

At the mucosal surface, the development of protective immune responses against *T. colubriformis* has been analysed through laproscopic biopsy or sequential necropsy (McClure et al., 1992; Rowe et al., 2009) with tissue or contents sampled through indwelling abomasal or intestinal fistulae (Jones et al., 1994; Greer et al., 2008; see Fig. 2 schematic). The expression of GIN immunity in ruminants is consistent with a T helper 2 (Th2) response with production of sensitized CD4+ lymphocytes expressing and secreting interleukins IL5, IL13 and TNFα (Pernthaner et al., 2005b, 2006; Ingham et al., 2008). Resistance to helminths in ruminants is associated with increasing levels of parasite-specific IgG1, IgA, IgE (Smith et al., 1983; Bendixsen et al., 2004; Pernthaner et al., 2005, 2006; Shaw et al., 2009; Hein et al., 2010) mucosal mast cells (MMCs) and eosinophils (Stewart, 1955; Emery, 1996; Emery et al., 1993; Hein et al., 2010). These responses prevent L3 establishment (Jones et al., 1994; McClure, 2000; Emery and Beveridge, 2015), suppress egg production, and remove adult worms (McClure et al., 1992). The levels of MMC sensitisation in the small intestine are highest around the *T. colubriformis* establishment site (first 5 m), diminishing distally (Bendixsen et al., 1995), so that paralysed worms may recover and establish further down the GI tract (Harrison et al., 1999). However, unequivocal evidence of a protective immune response against *T. colubriformis* has only been demonstrated for the carbohydrate larval antigen (CarLA) found on many nematode L3s. The CarLA response, which prevents L3 establishment, can be measured and is speculated to avoid "immune-mediated scouring", an untoward accompaniment to *T. colubriformis* immunity in some Australian and NZ flocks (Williams et al., 2010). This appears associated with eosinophilia in the intestinal mucosa (Larsen et al., 1995) and a conventional MMC-mediated rejection response.

2.2.2. Extrinsic inflammation and immunity to T. colubriformis

Several observations suggest that the induction of non-specific "allergic" inflammation in the small intestine may compromise *T. colubriformis* L3 establishment by up to 50%. These include lectin-containing navy beans in diets or i.p. inoculation of liposomes (D. Emery, unpublished data), oral dosing with attenuated *Salmonella typhimurium* aroA, and supplementation of diets with molybdenum or n-6 or neutral lipids (McClure, 2000).

2.2.3. Immunity to T. circumcincta

As observed with trickle infections with other GIN, immunity in lambs, dosed daily with 1000 *Tel. circumcincta* L3, develops from 4 weeks p.i. (Seaton et al., 1989), effectively regulating worm growth and the control of worm establishment/survival. Regulation of worm size and fecundity is strongly or causally associated with IgA especially against L4 (Stear et al., 1995; Martínez-Valladares, 2005; Strain et al., 2002). Eosinophil number is also inversely associated with worm length and fecundity (Stear et al., 2002), and eosinophils and IgA additively reduce worm growth and fecundity. Eosinophils have been implicated in resistance to infection with *H. contortus* (Meeusen and Balic, 2000; González et al., 2011) and inoculation of soluble extracts of *H. contortus* L3/*T. colubriformis* L3 into the mammary gland of immune and non-immune ewes elicits, within 24 h, an exudate comprising >90% eosinophils (Adams and Colditz, 1991).

The other major mechanism of resistance to *Tel. circumcincta* is IgE and MMC-mediated control of establishment and survival (Stear et al., 1995), alongside activation of pro-inflammatory Th17 cells in the mucosa (Gossner et al., 2012). IgE activity against iL3 seems to be most important (Sinski et al., 1995), preferentially targeting defined molecules (Murphy et al., 2010).

2.3. Epidemiological and genetic influences on GIN immunity

Mucosal immune mechanisms are influenced by physiological factors such as age, liveweight, pregnancy/lactation and sex, and external factors such as nutrition and stress (McClure, 2000;

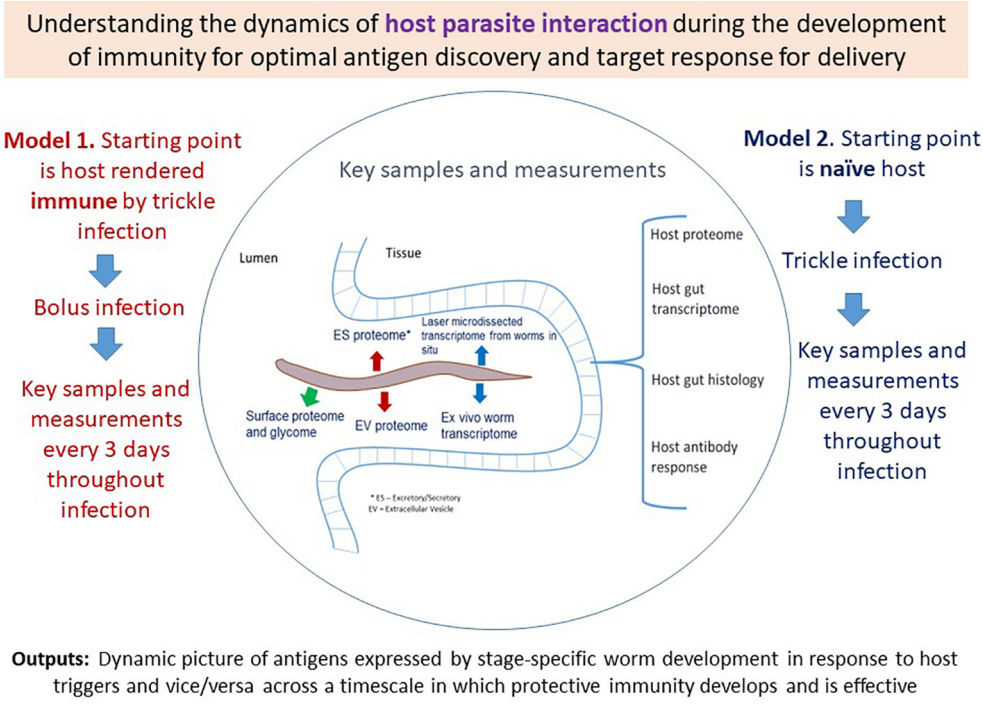

Fig. 2. Expanded schematic process for analysis of the dynamic development of host immunity during parasite maturation in vivo and ex vivo. The process aims to identify the induction of protective host responses and the parasite antigens, stages or metabolic activities which induce protection. EV, extracellular vesicles; ES, excretory secretions.

Coop and Kyriazakis, 2001). Conventional immunity to scour worms is transient and requires relatively constant ingestion of GIN to be maintained, as exemplified by volatility in anti-CarLA IgA titres (Shaw et al., 2012) and the slow acquisition of age-related resistance (Adams, 1993). Consequently, seasonal fluctuations in the levels of pasture L3s, as well as drenching regimes, delay the development of immunity in young lambs (Barger, 1988; McRae et al., 2015). Liveweight (nutrition/birth weight) also presents an issue for GIN immunity, with a weaning weight of 23 kg the critical minimum for optimal survival rates and production of Merino weaners in the face of GIN challenge (McClure and Emery, 2007).

Resistance to GINs is a complex genetic trait influenced by many genes and multiple non-genetic effects such as nutrition and prior exposure. The heritability of a single FWEC generally ranges between 0.1 and 0.4 which is sufficiently high to justify a selective breeding programme. Multiple FWECs improve the heritability. Alternative markers such as packed cell volume (Albers et al., 1987), parasite-specific IgA (Strain et al., 2002) and salivary IgA against CarLA (Shaw et al., 2012, 2013) may be cheaper to assay and similarly heritable.

Several studies have demonstrated the feasibility of selective breeding for resistance (Bisset et al., 2001; Woolaston and Windon, 2001; Karlsson and Greeff, 2006), with current application in commercial flocks in Australia, New Zealand and the UK. The major concerns are balancing the relationship with productivity and managing the transition to genomic selection. Mrode et al. (2018) recommend a cost-benefit analysis for farmers in developing countries to decide the most appropriate method of selection but these also apply to sheep breeders with extensively managed flocks.

3. Vaccine design: adjuvants and immunostimulants for ruminants

The variety of adjuvants (including "immunostimulants" and "immuno-enhancers") available to veterinary vaccinologists exceeds the limited array for human vaccines due to the various degrees of tissue irritability from many preparations. For the rational formulation of vaccines it is essential to understand the chemistry, properties and activity of the various adjuvants required to induce the desired protective response(s). For ruminants, this field is moving from empiricism to precision, as understanding of immunity develops.

3.1. Conventional adjuvants

Conventional veterinary adjuvants are either oil-based emulsions or other compounds, or combinations of both. The "type" of oil influences the extent of tissue reactions, with muramyl-dipeptide (MDP) in FCA causing large lesions which are not correlated with antibody titres (Stewart et al., 1985). More recent oil-based emulsions in the Montanide series (https://www.sepic.com/montanide-range) are less irritating with more consistent immunostimulation. Oil-based emulsions provide slow release of antigen over months; removal of the injection site and regional lymph node up to 3 months after vaccination caused significant reductions and persistence of antibody (Lascelles et al., 1989). Indeed, it was possible to achieve a heightened, antigen-specific, IgG response by inoculating an unrelated soluble antigen into an oil-based granuloma for up to 3 weeks after the initial s.c. injection (D. Emery, unpublished data). The depot, slow-release effect was consistent with a delayed but prolonged IFN-γ response from the draining lymph node, compared with shorter-acting adjuvants such as QuilA (saponin, QS21), dextran sulphate (DXS) and dodecyl-ammonium bromide (DDA) (Emery et al., 1990).

A range of adjuvants has been evaluated in sheep (Tables 1, 2). Most popular and well-characterised are the saponins (QuilA, QS21), alhydrogel (AH) and DXS. Quil A and DXS given subcutaneously are acutely inflammatory, giving rise to short, sharp rises in antibody and IFN-γ responses (Emery et al., 1990). Given that AH did not elicit IFN-γ production after inoculation or from primed lymphocytes (Emery et al., 1990), this adjuvant was presumed to elicit a protective Th2-type response (Emery, 1996). To stimulate ovine mucosal immunity, i.p. inoculation of incomplete Freund's adjuvant (IFA) was shown to stimulate intestinal immune responses (Beh and Lascelles, 1981), inducing widespread, ectopic, lymphoid aggregates in the mesentery.

The empirical nature of adjuvant use in ruminant GIN vaccine development is reflected in the lack of data directly comparing the effect of different adjuvants. Where comparisons exist, the results are inconsistent. For example, using a native extract of *H. contortus,* HcsL3, with Aluminium Phosphate induced significant levels of protection against *H. contortus* challenge, but the effect was abrogated when Quil A was added (Piedrafita et al., 2013). For Hc23, protection was similar in groups vaccinated with the antigen in Aluminium Hydroxide or Quil A, although Quil A alone conferred some level of protection (González-Sánchez et al., 2018). In contrast, vaccination of cattle against the abomasal parasite *Ostertagia ostertagi* with either native aspartyl proteinase (ASP) or ES-thiol induced protection when formulated with Quil A but not with $Al(OH)_3$ (Geldhof et al., 2004; Gonzales-Hernandez et al., 2016). Thus it appears that Th2-promoting adjuvants based on aluminium salts are not always associated with optimal protection, necessitating empirical testing and comparison of adjuvants.

3.2. Alternative delivery strategies and formulations for mucosal immunity

Substantial, and largely unsuccessful, efforts have been made to develop adjuvants and/or delivery systems that can induce mucosal immune responses local to the site of infection. The most effective way is by administration of vaccines onto the mucosal surface where antigen is primarily sampled by specialised cells (M-cells or goblet cells) which deliver antigen to underlying dendritic cells (DCs). In the case of M-cells, these localise to the follicle-associated epithelium (FAE) overlying mucosa associated lymphoid tissue (MALT). DCs present antigen to T cells within MALT or lymph nodes draining the mucosal site, to initiate the adaptive immune response (Neutra et al., 2001). During this process, expression of tissue-specific adhesion molecules and chemokine receptors are induced on activated T and B cells which allow lymphocytes to recirculate to mucosal sites. In contrast, vaccines delivered via s.c. or i.m. routes do not generally confer mucosal homing on lymphocytes and therefore are less efficient in inducing mucosal immune responses (McClure, 2008).

Mucosally-applied antigens face a formidable array of host defences: they may be diluted in mucosal secretions or trapped in mucus, excluded by epithelial barriers or agglutinating antibody and, in orally administered vaccines, face large dilutions within the rumen and degradation in the abomasum (McNeilly et al., 2008). Furthermore, uptake of antigens in the intestine often results in immune tolerance (Mayer and Shao, 2004). Mucosal vaccines must therefore deliver adequate levels of antigen across the mucosal barrier as well as activate appropriate signalling events to induce adaptive immunity and not immune tolerance.

3.2.1. Route of delivery

Mucosally-primed lymphocytes also home to distant mucosal sites, leading to the concept of the "common mucosal immune system" (McClure, 2000). Given the challenges associated with oral vaccines, the common mucosal immune system may be exploited

Table 1
Summary of vaccine trials for *Teladorsagia circumcincta* using native material and recombinant proteins.

Extract/Antigen	Dose	Route of vaccination	Adjuvant	% redn. (eggs)	% redn. (worms)	Reference
Mixed larval stage metabolites and ES from *in vitro* culture	300,000 larval equivalents	i.m.	FCA/IFA	81	44	Rose, 1976
		Orally (6 mth lambs)	none	76	46	
		i.m. (9 mth lambs)	FCA/IFA	0	7	Rose, 1978
		i.m. (3 mth lambs)	FCA/IFA	57	60	Rose, 1978
			none	47	48	
Live mixed stage larvae from *in vitro* culture	50,000 larvae	s.c. (6 mth lambs)	none	37	44	Rose, 1976
Macerated worms L4/L5 from *in vitro* culture	30,000 larvae	i.m. (6 mth lambs)	FCA/IFA	44	66	Rose, 1978
L3 soluble somatic proteins	~750 μg	s.c. (5 mth lambs)	$Be(OH)_2$	–[a]	32	Wedrychowicz et al., 1992
Detergent (CTAB) extract of xL3 surface	~750 μg	s.c. (5 mth lambs)	$Be(OH)_2$	–[a]	72	Wedrychowicz et al., 1992
Detergent (CTAB) extract of xL3 surface	~750 μg	s.c. (5 mth lambs)	FCA/IFA	–[a]	31	Wedrychowicz et al., 1992
L3 antigen precipitated by IgA from lambs immunised with CTAB extract of xL3 surface	~875 μg	s.c. (5 mth lambs)	$Be(OH)_2$	–[a]	72[b]	Wedrychowicz et al., 1995
L4 Con-A-binding fraction of a detergent-soluble membrane extract	34–48 μg	i.m. (4 mth lambs)	Quil A	29	–[a]	Halliday et al., 2012
Oc-gal-GP (Peanut and ConA lectin binding fractions from Triton X-100 extracts)	140 μg	i.m.	Quil A	28	–[a]	Smith et al., 2001
8 rec. antigen cocktail: Tci-APY-1; Tci-ASP-1; Tci-CF-1; Tci-ES20; Tci-MEP-1; Tci-MIF-1; Tci-SAA-1; Tci-TGH-2	50 μg each protein	s.c. in lambs 3-7 months old at first vaccination	Quil A	70	55	Nisbet et al., 2013, 2019
				58	56	
				0	15	
				14	3	
				49	67	
				52	64	
				0	46	
				47	74	
				30	50	
		s.c. in pregnant ewes	Quil A	44	ND[c]	Nisbet et al., 2016
4 rec. antigen cocktail: Tci-APY-1; Tci-CF-1; Tci-ES20; Tci-MEP-1	50 μg each protein combined	s.c. (4.5 mth lambs)	Quil A	0	0	Nisbet et al., Unpublished data
4 rec. antigen cocktail: Tci-ASP-1; Tci-MIF-1; Tci-SAA-1; Tci-TGH-2	50 μg each protein combined	s.c. (4.5 mth lambs)	Quil A	0	0	Nisbet et al., Unpublished data
2 rec. antigen cocktail: Tci-APY-1; Tci-MEP-1	50 μg each protein combined	s.c. (6 mth lambs)	Quil A	36	21	Nisbet et al., 2019
				3	34	
2 rec. antigen cocktail: Tci-mAPY-1[d]; Tci-MEP-1	50 μg each protein combined	s.c. (6 mth lambs)	Quil A	43	52	Nisbet et al., 2019

[a] Data not presented or not enumerated in manuscript.
[b] Note, ovalbumin also gave a 55% reduction in this experiment when administered with the same adjuvant.
[c] ND, not determined (ewes not euthanased).
[d] Loss of function mutant of Tci-APY-1 redn., reduction; mth, month; IFA, Incomplete Freund's adjuvant; $Be(OH)_2$, Berrylium hydroxide; rec., recombinant.

Table 2
Results of vaccine trials with *Trichostrongylus colubriformis* (Tc) extracts and antigens.

Extract/antigen	Dose	Route of vaccination	Adjuvant	% reduction (eggs)	% reduction (worms)
Tc L3 Homogenate[a]	30,000–50,000 L3[a]	JPP/rectal	Cellulose	49–53	30
			Chitosan	10–25	10
Post-exsheathment antigen (PEA)	100	IPP/IP	Quil/AH	34	48
	350	IPP/IP	AH	32	nd
Soluble Protein *Tc*L3[a]	5 mg	rectal	Cellulose	0	0
	1 mg	I/JPP	AH	12	10
*Tc*L3 pellet[a]	5 mg	rectal	Cellulose	0	0
Adult ES antigens	100 μg	IP	AH	52	47
ES 94 kDa	50 μg	IPP	AH	<30	<30
ES 11 kDa	50 μg	IPP	AH	<30	<30
ES 11 + 17 kDa or + 30 kDa	100 μg	IPP	AH	40	30–56
ES 30 kDa	50 μg	IPP	AH	<30	<30
ES recombinant 17 kDa	250 μg	SC × 2	Quil A	33	67
	100 μg			42	50
	33 μg			52	26
	10 μg			25	18
	3.3 μg			0	20
	100 μg	IP × 2	AH	46	32
			Quil A	0	ns
			IFA	15	ns
			AH-Quil A	24	ns
	1 mg	IPP × 2	Aqueous	34	3
			AH	43–62	24–57
			Quil A	42–53	26–41
			Dextran sulphate	66	37
			Alginate	39	28
			Fructose-1-P	57	13
			IFA	41	19
			Cellulose	37	47–71
	1 mg	Rectal PP × 2	Cellulose	<30	<30
			Chitosan	<30	<30
	1 mg	Pharyngeal LN ×2	cellulose	0	0
ES recombinant 37 kDa	20–50 μg	SC	Quil A	<25	<25
	50 μg	IP	Aqueous	Ns	Ns
			IFA	50	nd
			AH	46	nd
			Quil A	37–44	nd
			Dextran (DXS)	ns	nd
	100 μg	IPP-mucosa	Dextran-AH	36	All
	500 μg	JPP-mucosa	IFA	16	<30
	100 μg	Rectal PP	AH	18	
			Quil A	42	
			AH	11	
			PLG Microspheres	0	
			Quil A	29	

Data from McClure (2008)[a] and Emery, McClure and Wagland (unpublished data).
jpp/ipp, jejunal and ileal Peyers Patches; AH, aluminium hydroxide (alhydrogel); IFA, incomplete Freunds adjuvant (Montanide: Marcol 52); PLG, poly-lactide-glycolide microspheres.
Ipp, jpp were direct injections during laparotomy while rectal and mucosal routes involved delivery of vaccine to the luminal surface of the epithelium (McClure, 2000, 2008).

to induce mucosal immune responses local to the GINs by delivering antigens to accessible mucosal sites such as the terminal rectum or nasal cavity, which are both rich in MALT. Several trials where *T. colubriformis* antigens in either cellulose or chitosan gels were delivered intra-rectally in sheep conferred some degree of protection against subsequent challenge (McClure, 2008). Rectal immunisation induced parasite-specific antibodies in the jejunum which correlated inversely with FWECs and worm burden, providing evidence of a protective, common mucosal immune response.

Intra-nasal vaccination of sheep with PP2Ar (a recombinant part of the catalytic region of serine/threonine phosphatase 2A from the hookworm *Angiostrongylus costaricensis*) together with *Escherichia coli* bacterial cell walls induced protection against *H. contortus* and *Tel. circumcincta,* and intra-nasal vaccination of pigs and mice with recombinant *Ascaris suum* antigens reduced numbers of larvae recovered from the lung (Tsuji et al., 2001, 2003, 2004). These studies demonstrate the potential of intra-nasal vaccination to control GINs, although *A. suum* vaccines could operate in the intestine, lung or on migratory stages.

3.2.2. Live vectors

The efficacy of live vectors in mucosal vaccines is largely due to their ability to survive the luminal environment, actively invade mucosal tissues and trigger appropriate immune responses. For nematodes, most work on live mucosal vaccines has been conducted using attenuated strains of *Salmonella typhimurium* in mice. In these studies, oral or intra-nasal delivery of attenuated *S. typhimurium* expressing a number of *Trichinella spiralis* antigens elicited robust Th1/Th2 and mucosal IgA, and conferred protection against subsequent *T. spiralis* infection (Yang et al., 2010; Pompa-Mera et al., 2011; Liu et al., 2015; Wang et al., 2016). However, there are some concerns over safety and stability, and their efficacy in the face of pre-existing immunity to the vector.

3.2.3. Particulate delivery systems

Formulation of antigens into particles should protect the antigen from degradation and increase uptake by MALT. Enhanced uptake of microparticles is largely by M-cells which efficiently take up particles of <1 μm diameter. Little research has been conducted on the use of microparticles in nematode vaccines but, in mice, oral deliv-

ery of *T. spiralis* antigens incorporated into methacrilic acid copolymers successfully induced mucosal IgA responses and protection against parasite challenge, indicating the potential utility of the approach (Dea-Ayuela et al., 2006). Microparticles have been tested in sheep including poly(D,L-lactide-co-glycolide) microparticles to deliver *Toxoplasma gondii* antigens intranasally (Stanley et al., 2004), and ISCOMs®, 40 nm nanoparticles, to deliver viral antigens orally or intranasally (van Pinxteren et al., 1999; Coulter et al., 2003). Mucosal 'targeting' peptides, which facilitate transcytosis of conjugated molecules across the sheep intestinal epithelium to DCs in the lamina propria and Peyer's patches, may enhance intestinal microparticle uptake by incorporating these targeting peptides into particulate delivery systems (Kenngott et al., 2016).

3.2.4. Mucosal adjuvants

Induction of adaptive immune responses to inert mucosally-delivered antigens usually requires co-delivery of mucosal adjuvants e.g. Cholera Toxin (CT) and *E. coli* heat-labile toxin (LT). Only CT has been tested in sheep and induced mucosal IgA following rectal and intranasal delivery (Jacobs et al., 1999; Premier et al., 2004; Stanley et al., 2004). Chitosan gels used to deliver *T. colubriformis* antigens rectally possess adjuvant properties (McClure et al., 2008), and ISCOMs® have adjuvant properties as they are largely from the adjuvant Quil A (Morein et al., 2004). Toll-like receptor (TLR) ligands such as bacterial flagellins (Lee et al., 2006; Meeusen et al., 2013) and CpG (Gallichan et al., 2001) have also been successfully used as mucosal adjuvants, but not yet in sheep.

3.3. Induction of Th2 responses in sheep

In sheep models of allergic asthma, SC immunizations with ovalbumin (Abraham, 2008; Abraham et al., 1983), peanut allergen (Van Gramberg et al., 2012) and dust mite allergen (HDM), with alum adjuvant (Bischof et al., 2003) have successfully induced antigen-specific IgE. In the dust-mite model, 50 mg of HDM resulted in approximately 60% of sheep developing high levels of HDM-specific serum IgE. Experimentally sensitized sheep segregated into high and low IgE responders to HDM, and those with high specific IgE serum titres (allergic sheep) generated an elevated and prolonged broncho-alveolar lavage (BAL) containing eosinophilia when challenged with HDM (Bischof et al., 2003). This would suggest that if antigen-specific IgE responses provided protection against GIN, these protocols could be used to induce Th2 responses in sheep mucosa.

Many parasite infections induce a Th2 response with elevated IgE (see Shaw et al., 2009). Following *T. colubriformis* challenge, immune animals had higher levels of total IgE in intestinal lymph than non-immune sheep, low FWEC sheep had higher concentrations of total IgE than high FWEC sheep, and field-primed animals had higher total IgE levels than GIN-naıve animals, arising from stage-specific induction of IgA and IgE responses in regional mesenteric lymph nodes (MLNs) (Pernthaner et al., 2006). Similar observations have been made following *Tel. circumcincta* challenge where IgE responses to L3 but not adult antigens were detected in abomasal lymph samples from immune versus non-immune sheep (Huntley et al., 1998). Serum IgE obtained from high and low FWEC lambs bound to western blotted *T. colubriformis* L3 antigens of 40 (galectin), 31 (Aspin) and 21 kDa (Shaw et al., 2013; Bendixsen et al., 2004). Levels of specific IgE to *T. colubriformis* L3-excretory-secretory antigens (ES) was heritable ($h^2 = 0.36$) and showed a negative genetic correlation with FWEC (ca −0.31; Shaw et al., 1999). IgA titres to CarLA were both moderately heritable (0.28 ± 0.10) and negatively correlated with FWECs (−0.57 ± 0.20; Shaw et al., 2013).

Induction of Th2 responses has been attempted by various routes, but the precise role of IgE in protection is still unresolved. What is clear is that:

- antigens on parasites recognised by IgE or IgA from immune animals do not possess intrinsic "allergic" potential – they do not readily generate IgA or IgE when presented in purified or recombinant- adjuvanted forms;
- formulations which induce Th2 responses in rodent models do not reliably extrapolate to ruminants; and,
- vaccine formulations which induce mucosal Th2 responses in ruminants are not readily available.

While induction of Th1 immunity is largely understood, the mechanisms underlying initiation of Th2 immunity have been unclear until recently. Studies in murine nematode infections indicate a critical role for epithelial tuft cells in the initiation of Th2 immunity to GIN (Gerbe et al., 2016; Howitt et al., 2016; von Moltke et al., 2016). Tuft cells appear to be the primary source of the Th2 alarmin, IL-25, which activates innate lymphoid cells (ILC); ILC2-derived IL-13 acts in a positive feedback loop to expand tuft cell numbers. Once sufficient IL-25 and IL-13 are generated, Th2 immunity is induced. Putative tuft cells have been identified in sheep and expand during *Tel. circumcincta* infection in a similar manner to that observed in mice (K. Hildersley, C. Britton, T.N. McNeilly et al., unpublished data). As tuft cells are chemosensory, it is possible that their activation to produce IL-25 is through sensing 'helminth-associated molecular patterns', or molecules indirectly induced in the gut lumen following parasite infection. If identified, these tuft cell activating molecules could be used as adjuvants to drive more effective Th2 immunity.

3.4. Immune modulators for redirection of systemically induced immune responses to mucosal surfaces

Specific adjuvants for systemically delivered vaccines which target the immune response to mucosal sites have not yet been widely exploited in small ruminants. However, vitamin D3 in systemically delivered subunit vaccines induces mucosal antigen-specific IgA responses in mice, pigs and cattle (Daynes et al., 1996; Van der Stede et al., 2001; Vilte et al., 2011). Other examples include TLR ligands, poly I:C (TLR3 ligand), lipopolysaccharide (LPS), (TLR4 ligand), and flagellin (TLR5 ligand), which induce mucosal IgA following systemic delivery (Enioutina et al., 2008; McNeilly et al., 2008,). TLR3 and TLR4 upregulated 1α-hydroxylase in DCs which converts circulating 25(OH)D3 to calcitriol, the active form of vitamin D3. Interestingly, TLR3 and TLR4 ligands were capable of inducing IgA to co-administered soluble antigens, whereas for the TRL5 ligand flagellin, IgA responses were directed towards flagellin but not the co-adminstered antigens (McNeilly et al., 2010), suggesting a need for physical linkage of antigen and TLR ligand for IgA induction. Exploiting vitamin D, either by direct inclusion into systemically delivered vaccines or incorporation of TLR ligands which upregulate vitamin D activity, or incorporating other TLR ligands into antigen-containing microparticles, may be a useful approach to enhance mucosal immune responses in nematode vaccines.

4. GIN vaccines for scour worms - antigen discovery and types of vaccines

There are five main categories of vaccines for the control of pathogens in humans and livestock:

(1) chemically abbreviated infections ("truncated" infections);
(2) pathogens attenuated by irradiation or culture (or use of closely-related, less pathogenic organisms);
(3) protein subunit/nucleic acid subunit vaccines – (native proteins, protein complexes or recombinant subunits);

(4) pathogens killed by chemicals or by heat;
(5) toxoids – inactivated compounds where these (rather than the organisms) cause disease.

The successful development of vaccines against GIN has focussed on the first three categories.

Examination of naturally-acquired immunity against the principal GINs of small ruminants indicates that the most vulnerable stage is iL3, through reduced establishment rates (Barger et al., 1985; Dobson et al., 1990) or larval development (Seaton et al., 1989). Expulsion occurs in a common time-frame and effector mechanism (Stewart, 1955; Emery et al., 1993; Harrison et al., 1999). The L3 stage of GIN parasites would be the most obvious target, negating the pathology associated with later stages of worm development and expulsion (Barker, 1975; McKellar, 1993; Stear et al., 2003; Greer et al., 2008; MLA, 2017). However, since low doses of iL3s can subvert established immunity (Smith et al., 1983; Dobson et al., 1990), information on protective immunogens from later stages of GINs is pivotal for rational vaccine development.

Natural immunity against GINs is rarely "sterile". Under natural conditions with low dose challenge, some worms establish even in immune animals (Smith et al., 1983). Three thousand to 4500 iL3s is considered a threshold to ignite the immune response (Dobson et al., 1990). Comparatively, GIN with developmental stages in tissues (i.e. *Tel. circumcincta, O. columbianum)* are more vulnerable to suppression/retarded development than those residing on the mucosa.

4.1. Chemically abbreviated (truncated) infections

Chemically abbreviated infections have been used to investigate protective immunity. Repeated rounds of *T. colubriformis* infection (3× infections of 30,000 iL3) generated solid resistance to homologous L3 and adult worms in hoggets, rejection occurring within 3–5 days (Emery et al., 1992a). Sheep given similar 4 × 7 days and 4 × 10 days larval infections rejected *T. colubriformis* L3 challenge but rejected only 50% of adult worms while sheep given 4 × 4 days infections remained fully susceptible. This indicated that stage-specific antigens produced by early L3 and L4 stages effectively immunized sheep, while sheep immunised with adoptively transferred adult worms took 7–10 days to reject the iL3 challenge, presumably when adult antigens were produced (Emery et al., 1992b). Such approaches proved less successful in 1 month old lambs (McClure et al., 1998). However, similar truncated infections also induced >90% protection against *T. colubriformis* ingested from pasture (Stankiewicz et al., 1996; Harrison et al., 2003), with "immunity-induced" weight loss associated with increased levels of gut eosinophils (Stankiewicz et al., 1996). This approach was unsuccessful against homologous *Tel. circumcincta* challenge (Harrison et al., 2003) but successful against *H. contortus* (Robinson et al., 2010).

McClure (2008) administered 18 doses of 1200 *T. colubriformis* L3 (in cellulose gel; three per week for 6 weeks) to the rectal lymphoid tissue and mucosa, generating >88% protection against a challenge infection of 30,000 L3, demonstrating additional routes of vaccination other than oral or IP (see Section 4.2).

4.2. Radiation-attenuated vaccines

Following the successful immunisation of calves against bovine lungworm (*Dictyocaulus viviparus*) using two doses of 1000 irradiated L3s (Jarrett et al., 1959; later Dictol®), Dineen et al. (1978) used irradiated *T. colubriformis* L3s to select Merino weaner lambs into resistant and susceptible genotypes. Successive generations of the model were used to elucidate the mechanisms of GIN resistance. While this approach successfully segregated response phenotypes, the 2 × 20,000 L3 vaccination protocol was unrealistic for the field. Immunisation of sheep >7 months old with irradiated *H. contortus* L3 or *T. colubriformis* L3 conferred high levels of protection against homologous challenge (Jarrett et al., 1961; Dineen et al., 1977; Smith and Christie, 1978; reviewed by Bain (1999)).

In trials with *Tel. circumcincta*, 8 month old Greyface-Suffolk cross sheep were immunised with two doses of 10,000 gamma-irradiated iL3 4 weeks apart and then challenged, 4 weeks later, with a bolus of 10,000 non-irradiated iL3ss (Smith et al., 1982). Four weeks after the challenge dose, adult worm burdens of immunised sheep were 60% lower ($P < 0.05$) than controls and peak FWEC was reduced by 65% (Smith et al., 1982). When irradiated worms were removed by anthelmintics 1 week before challenge, the protective effect was lost, demonstrating the need for concomitant infection with the irradiated larvae for protection (Smith et al., 1982). Similar levels of protection have also been demonstrated by immunising 3–5 month old lambs with two oral doses of 50,000 *Tel. circumcincta* L3s attenuated by UV irradiation Wedrychowicz et al. (1992).

4.3. Native antigen-based vaccines

4.3.1. Parasite extracts

Barbervax® Gut membrane preparations of *Haemonchus* containing glycoprotein complex H-gal-GP and aminopeptidase H11 have consistently conferred protection against homologous challenge (Le Jambre et al., 2008). These antigens, in microgram quantities, conferred high levels of protection, allowing the commercial exploitation of a native vaccine (reviewed in Nisbet et al., 2016a). The antigens present in Barbervax® are "concealed" so, in the absence of exposure-related boosting of the immune response, repeated vaccination is required to stimulate high antigen-specific circulating antibody levels. An advantage of these concealed antigens is that they can produce protective effects in situations where natural immunity is either weak or ineffective, such as in young lambs or in periparturient ewes (Le Jambre et al., 2008).

Teladorsagia circumcincta. Table 1 summarises trials using either native or recombinant *Tel. circumcincta* antigens. Immunisation of 5 month old Finn-Dorset male lambs with a detergent extract of exsheathed L3 (surface antigens) formulated with Beryllium hydroxide adjuvant, followed by challenge with a bolus of 50,000 iL3, resulted in parasite burdens which were significantly reduced (by 72%, $P < 0.01$) compared with non-immunised controls (Wedrychowicz et al., 1992, 1995). Somatic extracts of L3 in the same adjuvant gave only modest reductions in worm burden (30%) compared with control lambs (Wedrychowicz et al., 1992). In contrast, somatic extracts of L4/early L5, administered to 6 month old Dorset Horn lambs by repeated injection in FCA/IFA over a 3 week period, induced protection (66% reductions in adult worm burden, 44% reduction in FWECs) when challenged with a trickle infection of 5000 iL3s daily over a 15 day period (Rose, 1978).

Immunisation of 2 month old Suffolk-Greyface cross lambs with *H. contortus* H11 and H-gal-GP conferred no cross–protection against *Tel. circumcincta* (Smith et al., 2001). When 9 month-old Suffolk-Greyface sheep were immunised with lectin-binding integral membrane glycoproteins derived from adult *Tel. circumcincta*, with Quil A, and challenged with a bolus of 5000 iL3s *Tel. circumcincta*, worm burdens and FWECs were reduced by only 8% and 28%, respectively, compared with control, adjuvant-only recipients (Smith et al., 2001). As immunisation had induced high levels of circulating antibodies, it was concluded that adult *Tel. circumcincta* did not consume sufficient antibody for the gut antigen approach to be effective (Smith et al., 2001). Based on the hypothesis that L4 *Tel. circumcincta* induce more localized inflammation at the site

of infection than adult worms, and therefore are exposed to higher quantities of ingestible immunoglobulin, Halliday and Smith (2011) immunised 4 month old Scottish Mule lambs with lectin-binding integral membrane glycoproteins derived from L4 *Tel. circumcincta*, with Quil A, and challenged the lambs with either a bolus (5000 iL3) or trickle (500 iL3s daily for 10 days) *Tel. circumcincta* challenge. No significant reductions were observed in either FWECs or worm burden with either challenge method (Halliday and Smith, 2011).

Trichostrongylus colubriformis. Table 2 summarises vaccine trials with a range of antigens and preparations from *T. colubriformis*. From initial screens in the guinea pig model and the premise that surface or ES antigens shed from various parasitic stages contribute to protective immunity observed with trickle iL3s infections (Dobson et al., 1990), L3 homogenates, post-exsheathment antigen (PEA) and native adult ES antigens were employed. Animals were challenged with trickle infections of 1500–2000 L3s, 3 × weekly for 3 weeks. Larval homogenates and soluble proteins, PEA and post-extraction iL3s pellets in a variety of adjuvants failed to generate consistent protection (>57%) when administered either s.c., i.p., inoculated into intestinal Peyers Patches (JPP/IPP) or when in cellulose gels applied to the rectal mucosa (McClure, 2008). Throughout these trials, antigens incorporated into AH, which did not elicit IFN-γ (Emery et al., 1990), generated higher levels of immunity than other adjuvants.

4.3.2. ES antigens

Trichostrongylus colubriformis. Adult *T. colubriformis* produce ~1 mg of ES protein per 25,000 worms daily (unpublished observations). Early vaccine trials, using L3 soluble proteins (SpL3) or ES antigens from L3 or adult worms gave >90% reductions in worm counts in a guinea pig infection model (Rothwell et al., 1994). The protection was reduced if ES proteins were administered in FCA instead of AH (Wagland et al., 1996). Subsequent comparisons in sheep were less successful; worm burdens reduced by 30–70% when adult ES material (1 mg/dose) was used (see Emery, 1996; Table 2).

Teladorsagia circumcincta. Early vaccine trials using ES material from mixed stage *Tel. circumcincta*, administered in FCA/IFA to 9 month old cross bred lambs repeatedly over a period of 3 weeks followed by a trickle infection of 5000 L3 daily for 15 days, gave no reductions in either FWECs or worm count (Table 1) (Rose, 1978). In contrast, when 3 month old Dorset Horn lambs were immunised with concentrated ES from mixed stage *Tel. circumcincta* (with or without FCA/IFA) and challenged by a trickle infection of 7500 L3s per day for 4 days, immunised lambs had between 48% and 69% fewer adult worms and reductions in FWECs of 47% to 60% compared with controls (Rose, 1978).

4.3.3. CarLA (Carbohydrate Larval Antigen)

Harrison et al. (2003) isolated a heat-stable 35 kDa carbohydrate antigen (CarLA) when *T. colubriformis* L3 extracts were probed with sera from GIN-immune sheep and /or genetically resistant animals. The antigen was shared by a range of GIN, including *H. contortus, Tel. circumcincta* and *Nematodirus.* CarLA was poorly immunogenic, and a range of coupling agents were examined to attempt to boost its immunogenicity. Although the antigen was not tested directly as a vaccine, indirect evidence of its putative protective capacity was inferred from the inhibition of establishment of *T. colubriformis* which had been pre-incubated with immune mucus before dosing to sheep (Harrison et al., 2003). Interestingly, while anti-CarLA Ig in immune mucus inhibited *T. colubriformis* establishment, *H. contortus* and *Tel. circumcincta* were virtually unaffected (Harrison et al., 2008). It was later demonstrated that *H. contortus* possessed different isoforms of CarLA, and that mucus isolated from sheep immunised with *Haemonchus* or *Teladorsagia* CarLA did not generate high levels of anti-CarLA antibodies (Harrison et al., 2008). However, titres of anti-CarLA IgA, although transient, are indicative of worm immunity (Shaw et al., 2012).

4.4. Recombinant antigen vaccines

The development of recombinant subunit vaccines for the protection of small ruminants against GIN has been, until recently, relatively unsuccessful (McClure, 2009; Nisbet et al., 2016a; Matthews et al., 2016; Stutzer et al., 2018). Recent work to develop a recombinant subunit vaccine for *Tel. circumcincta* has shown some promise in both lambs (Nisbet et al., 2013) and periparturient ewes (Nisbet et al., 2016b).

4.4.1. Teladorsagia circumcincta

Previous evidence of the underlying immune mechanisms acting against the parasite (Smith et al., 1985, 1986, 1987), was used to select L3 and L4 ES proteins which were targets of local IgA responses in immune sheep. Bioinformatic analysis (Nisbet et al., 2008) also identified an immunogenic homologue of a protective antigen of the canine hookworm, *Ancylostoma caninum* (Ac-SAA-1 (Zhan et al., 2004; Nisbet et al., 2009)); and potential immunosuppressive molecules released by the parasite were also identified (McSorley et al., 2009; Nisbet et al., 2010, 2011). The eight vaccine candidates identified using this tripartite approach were prepared as a recombinant cocktail vaccine and formulated in Quil A. In Texel-cross (6–7 month old) lambs, in two separate trials, this prototype cocktail vaccine reduced cumulative FWECs (cFWECs) by 70% and 58% compared with control lambs which received adjuvant only (Nisbet et al., 2013). The parasite challenge in each of these experiments was a 4 week trickle infection and, during the period of peak worm egg shedding, vaccinated lambs shed 92% and 73% fewer eggs than control lambs. Vaccinates had 75% and 56% lower mean adult nematode burdens than controls (Nisbet et al., 2013).

Using the same vaccine to immunise lambing ewes resulted in a 44% reduction in mean cFWECs levels compared with ewes given adjuvant only (Nisbet et al., 2016b). More recently, Nisbet et al. (2019), used data from five independent vaccine trials with this cocktail vaccine, to simplify its antigenic complexity. This meta-analysis demonstrated statistically significant reductions in FWECs and worm burden in vaccinated sheep across trials ($P = 0.009$ and $P < 0.0001$, respectively). Relationships between antigen-specific antibody (IgA; total IgG; IgG1 and IgG2) levels, antibody avidity and parasitological parameters of efficacy were analysed for each of the eight proteins in these trials. The strongest correlations between percentage reduction in cFWEC and avidity were obtained for Tci-APY-1 (a calcium-dependent apyrase). The inclusion of Tci-APY-1 in a simplified vaccine was further supported by the observation that serum and mucosal IgG and IgA of control (parasite challenged) lambs strongly recognised Tci-APY-1 as well as a further vaccine antigen, Tci-MEP-1. A two-protein vaccine (Tci-APY-1 and Tci-MEP-1) was tested in a direct comparison with the original eight-component vaccine (Nisbet et al., 2019). In the same experiment, a further group was immunised with Tci-MEP-1 plus a mutated form of Tci-APY-1 (mTci-APY-1), which had no enzymatic activity. The rationale behind the inclusion of a loss-of-function mutant apyrase was that a previous study demonstrated that transient ATP release at the injection site is required for optimal efficacy of some adjuvants and that inclusion of an active apyrase into an adjuvanted influenza vaccine led to inhibition of innate and adaptive responses, presumably due to hydrolysis of extracellular ATP (Vono et al., 2013).

Across the timeframe of the trial, the FWEC levels of the eight-antigen recipients were consistently lower than those of the adju-

vant only control group ($P = 0.013$) and the mean cFWEC of the mTci-APY-1 and Tci-MEP-1 recipients was lower (43%), although not statistically significantly ($P = 0.079$), than that of the controls (Nisbet et al., 2019).

4.4.2. Trichostrongylus colubriformis

Several adult ES proteins expressed in *E. coli* or baculovirus (11, 17, 30, 37 and 94 kDa) have been investigated as protective immunogens in sheep (McClure, 2008; Frenkel et al., 1992; Table 2). A series of trials were undertaken, using adjuvant combinations and targeting mucosal delivery routes (IP, Peyer's Patches and rectal mucosa) to sensitise MMC and generate worm-specific IgG1, IgA and IgE (Emery, 1996; McClure, 2008). Table 2 lists much unpublished work to prevent repetition of past efforts. Overall, these antigens and protocols failed to generate >70% protection. However, there was little doubt that mucosal vaccination, especially through IPP and JPP using antigens in AH, effectively generated antigen-specific IgG1, IgA and IgE responses.

4.5. Novel expression systems

A major hurdle in vaccine development is expression of vaccine candidate genes in appropriate expression systems. Genes from *Tel. circumcincta* and *H. contortus* can be expressed in *Caenorhabditis elegans* and can rescue *C. elegans* mutant phenotypes, demonstrating correct folding and functional activity (Britton and Murray, 2006; Baker et al., 2012). While *C. elegans* may be appropriate for expression of some parasite proteins, our group were unable to express *H. contortus* vaccine antigen H11 or cysteine or aspartyl proteases in *C. elegans* in a form that can induce protection (Roberts et al., 2013). This suggests that a combination of antigens is required to induce immunity or, alternatively, specific glycans present on H11 and other vaccine antigens are needed and are not present on *C. elegans* expressed proteins. Detailed mass spectrometry comparison of glycans present on native *H. contortus* H11 and *C. elegans*-expressed recombinant H11 identified similar di- and trifucoslyated glycans but there was a lack of fucosylated LacdiNAc (LDNF) and Galα1-3GalNAc structures on *C. elegans* recombinant H11 (Roberts et al., 2013). The use of synthetic glycans as vaccines would help test the relevance of glycan structures to protection (Astronomo and Burton, 2010).

Where glycans and other post-translational modifications introduce conformational epitopes, these can be extremely challenging to reproduce in a recombinant expression system. One approach is to use commercially available random peptide phage-display libraries to identify short peptide epitopes that are bound by antibodies from immune animals. These phage-displayed peptides may represent primary protein sequence or more complex secondary or tertiary structural epitopes, and may even mimic non-peptide components such as carbohydrate moieties. Phage-displayed peptides have been used in the development of prototype vaccines against a range of parasites including *T. spiralis* (reviewed in Ellis et al., 2012). Preliminary work using antibodies from sheep immune to *Tel. circumcincta* to pan phage-displayed peptide libraries identified five peptide sequences which mimicked surface and/or glycan epitopes on exsheathed L3s (Ellis, 2014. Interactions of *Teladorsagia circumcincta* with the ovine immune system – mimicry and vaccine development. PhD Thesis, University of Edinburgh, Scotlan.). Positive correlations were demonstrated between peptide-specific IgA levels for these five peptides, and the percentage of inhibited L4s in the abomasum, whereas there were negative correlations between the levels of peptide–specific IgA and total nematode burden (Ellis, 2014, cited above).

Plant expression systems can produce high levels of protein in soluble, secreted form, technology to manipulate their glycosylation machinery is available and they are being tested for expression of parasite proteins, particularly those where folding and/or glycosylation are critical for function or immunity (Wilbers et al., 2017). Transgenic tobacco has been used to determine the structure and binding properties of nematode venom allergen-like proteins (VALs) (Asojo et al., 2018; Darwiche et al., 2018). A similar approach would be valid for testing expression, function and protective capacity of related ASPs and other vaccine candidates from scour worms.

5. Technological advances and opportunities (and how they can be used)

5.1. Current status of worm assays

There are limitations for the in vitro cultivation of GIN. While exsheathed *Haemonchus* L3 develop to L4 in simple medium, more complex conditions are needed for development to adults (Douvres, 1980; Stringfellow, 1986). Consequently, researchers have relied on supplies of iL3, or parasites at various stages of development ex vivo (eg. Rowe et al., 2008; McNeilly et al., 2017), to measure viability, egg production or comparative measurements of length or sex ratios (eg. Rowe et al., 2008; McNeilly et al., 2017). In vitro studies on immunity have relied primarily on the Larval Development Assay (LDA; Lacey et al., 1990) or paralysis of L3 motility in various "wriggle-ometers", with or without downstream 'omics' investigations (Jones et al., 1994; Hu et al. 2013,). RNA interference (RNAi) and functional validation studies have used short-term cultures, with assay of the target gene, its transcription or products (Kotze and Bagnall, 2006) or worm survival in vivo (Samarasinghe et al., 2011). Progress in this area would greatly assist vaccine development and antigen validation.

5.2. Antigen identification from genomic and transcriptomic data

Vaccine candidate identification has relied mainly on the use of antisera from immune or convalescent animals to screen either cDNA libraries or immunoblots for immunodominant antigens (e.g. Verkuylen et al., 1993; Redmond et al., 2006) or in immunoaffinity purification (e.g. Ellis et al., 2014). However, over the last decade information on the complete set of genes present in parasitic nematodes, the lifecycle stages in which these genes are expressed and detail of their putative functions, has greatly increased. This allows rational selection of potential vaccine targets including enzymes catalyzing unique metabolic pathways, secreted and surface proteins, and immunoregulatory molecules. Comparing genomes across species will establish a map of genes conserved across diverse nematodes, to help identify biological function and reveal potential pan-nematode targets.

5.2.1. Genomics and transcriptomics

Genomic data is available for a range of nematodes, including many of veterinary importance. A comparative genomics analysis of 56 nematode species, including *H. contortus* and *Tel. circumcincta*, has recently been published (International Helminth Genomes Consortium, 2019), supported by the 50 Helminth Genomes project (https://www.sanger.ac.uk/science/collaboration/50hgp). Progress has been helped by the reduced cost of sequencing, together with improved technologies and bioinformatics pipelines making it feasible to sequence the genome of GIN relatively quickly, although generating high quality assembled and annotated genomes is still challenging due to the large size of GIN genomes (approximately 300–700 Mb, representing an estimated 20,000 protein coding genes) and the high level of polymorphism within populations. Of the GIN considered here, most

advanced genome information is currently available for *H. contortus*, sequenced independently from UK and Australian isolates (Laing et al., 2013; Schwarz et al., 2013). This was achieved using short read sequencing technologies to provide short DNA sequences which are then assembled into contigs and scaffolded by mate pair data. Refinement of the initial *H. contortus* genome assembly has involved the use of long-read sequencing, optical mapping and intensive manual curation, enabling assembly at a chromosome level (Doyle et al., 2017). This assembly has been annotated with the inclusion of Iso-Seq data, which generates full-length cDNA sequences.

Technologies developed for *H. contortus* genome assembly can be applied to related GIN including *Tel. circumcincta* and *Trichostrongylus* species. *Tel. circumcincta* genome sequencing and assembly are in progress at the Wellcome Sanger Institute, UK and McDonnell Genome Institute, St Louis, USA. Genetic diversity in the *Tel. circumcincta* population, and strikingly large genome size, make this challenging. Genetic variation was previously observed for *H. contortus* and is compounded by polyandry, a phenomenon also reported for *Tel. circumcincta* (Redman et al., 2008; Gilleard and Redman, 2016). For *T. colubriformis,* genome sequencing is in progress at the United States Department for Agriculture (USDA; USA) (https://www.ars.usda.gov/research/project/?accnNo=431526). Current sequence data for GIN genomes can be accessed at the WormBase ParaSite website (https://parasite.wormbase.org/index.html), which allows comparison of gene sequences across species, as well as predicted functional information based on gene ontology (GO) data. However, the function of many nematode genes is currently unknown; notably 47% of nematode gene families lack any functional annotation (International Helminth Genomes Consortium, 2019). Genome sequences will allow identification of genes and pathways conserved across GIN, for rational selection as vaccine candidates.

Transcriptomic data are publicly available for some species of scour worms and these data are usually generated from the parasites at specific developmental stages, living in specific niches or, in some cases, from individual sexes. For *T. colubriformis*, transcriptomic data for adult (mixed sex) worms (Cantacessi et al., 2010) have been deposited in publicly accessible sites (http://www.nematode.net/ and http://research.vet.unimelb.edu.au/gasserlab/index.html). For *T. vitrinus* there is less publicly-available transcriptomic sequence; studies by Nagaraj et al. (2008) and Nisbet and Gasser (2004) used the same set of ~400 sex-specific adult expressed sequence tags (ESTs) in an early comparative transcriptomic study but few sequences have been added since then. In contrast to the paucity of EST data for some *Trichostrongylus* spp., there is more transcriptomic data available for *Tel. circumcincta* (Nisbet et al., 2008; Menon et al., 2012) and currently on http://nematode.net there are a large number of *Tel. circumcincta* genes for which RNAseq expression data are available. Additional, "niche-specific" transcriptomes are available: exsheathed L3s (xL3) exposed to abomasal extracts from immune and non-immune sheep (Halliday et al., 2012) and mucosal-dwelling (MD) versus lumen-dwelling (LD) L4 (McNeilly et al., 2017). In this latter study, transcripts significantly differentially expressed between MD and LD larvae were identified, of which the majority were up-regulated in MD larvae and encoded a suite of potentially immune-regulatory proteins produced by the parasite only when in intimate contact with the host, which might be exploitable as vaccine candidates (McNeilly and Nisbet, 2014).

For *H. contortus*, RNAseq expression data are publicly available (Laing et al., 2013). Of particular relevance to vaccine development, a female gut-specific transcriptome identified a suite of gut-expressed cysteine-type peptidase and cysteine-type peptidase inhibitors (Laing et al., 2013). Recent differential transcriptomic analysis in *H. contortus* has also demonstrated that worms surviving in sheep vaccinated with Barbervax® had an increased expression of genes encoding (non-Barbervax®) proteases and regulators of lysosome trafficking (Sallé et al., 2018). Both publications support previous research suggesting that gut proteinases, and their regulators, are strong vaccine candidates (reviewed in Nisbet et al., 2016).

5.2.2. Reverse vaccinology

The traditional design of vaccines was complemented by "reverse vaccinology" (RV) in the mid-late 1990s following the advent of whole genome sequencing (WGS) of pathogens (Fig. 3). The original RV approach used genomics, high throughput recombinant protein expression and screening in vivo for early antigen discovery. This process has since evolved to "reverse vaccinology 2.0" which adds the development of host-specific monoclonal antibody (mAb) production, B cell repertoire deep sequencing, proteomics and structure-based antigen design to the original approach (reviewed in Rappuoli et al., 2016).

In RV, vaccine candidates are initially identified using in silico algorithms (e.g. Vaxign, http://www.violinet.org/vaxign/; He et al., 2010; reviewed in Lew-Tabor and Rodriguez Valle (2016) to predict open reading frames (ORFs) of surface, membrane or secreted proteins from whole genomes. For this reason, a complete, assembled, annotated genome is the preferred starting point, although a deep stage-specific transcriptomic analysis for vaccines targeting specific developmental stages of GIN might be a reasonable proxy. These genomic/transcriptomic components of the classical RV approach are either already available (e.g. in the case of *Haemonchus contortus*), are in development (*Tel. circumcincta, T. colubriformis*) or are lacking (*T. vitrinus*) (recently reviewed by Jex et al. (2019)). It should be noted, however, that most *in silico* prediction algorithms have been developed with viral or bacterial pathogens, but not helminths, in mind. The other components of classical RV – capacity to produce recombinant proteins in high throughput, appropriate immunogenic and protective forms, and robust animal models for the testing of candidate antigens – are both available for testing of prototype vaccines against scour worms. Recently, significant progress has been made in the use of RV approaches to develop vaccines against the cattle tick (*Rhipicephalus microplus*) and the Australian paralysis tick (*Ixodes holocyclus*) (Lew-Tabor and Rodriguez Valle, 2016; Tabor, 2019). SophisTICKated anti-tick vaccines- cattle tick and Australian paralysis tick. Proceedings of the UK and International Veterinary Vaccinology Network Conference, London 9–10th January 2019) with the vaccine for the former having gone through 12 years of antigen discovery, bioinformatics, in vitro immune screening and in vitro tick feeding followed by seven challenge trials.

The large genomes of parasitic helminths and the potential that recombinant vaccine candidates may need to be expressed in eukaryotic systems for optimal efficacy both make the traditional RV route cumbersome for parasitic worms (Merrifield et al., 2016). Nevertheless, significant progress has been made in the use of RV to identify vaccine antigens from *Schistosoma mansoni* through in silico prediction of immunogenic epitopes of transmembrane proteins coupled with selection of these epitopes for high binding affinities for host MHC class II molecules (Oliveira et al., 2016; de Souza et al., 2018). Algorithms developed for human MHC molecules could potentially help identify immunogenic molecules from sheep parasites, although algorithms specific for small ruminant MHC molecules are likely to be more helpful.

Reverse vaccinology 2.0 is considerably more advanced in human vaccine development than in veterinary species. Because most licensed vaccines act by inducing protective antibody responses, most of the key breakthroughs for RV 2.0 have revolved around antibody technologies. The key elements of the approach

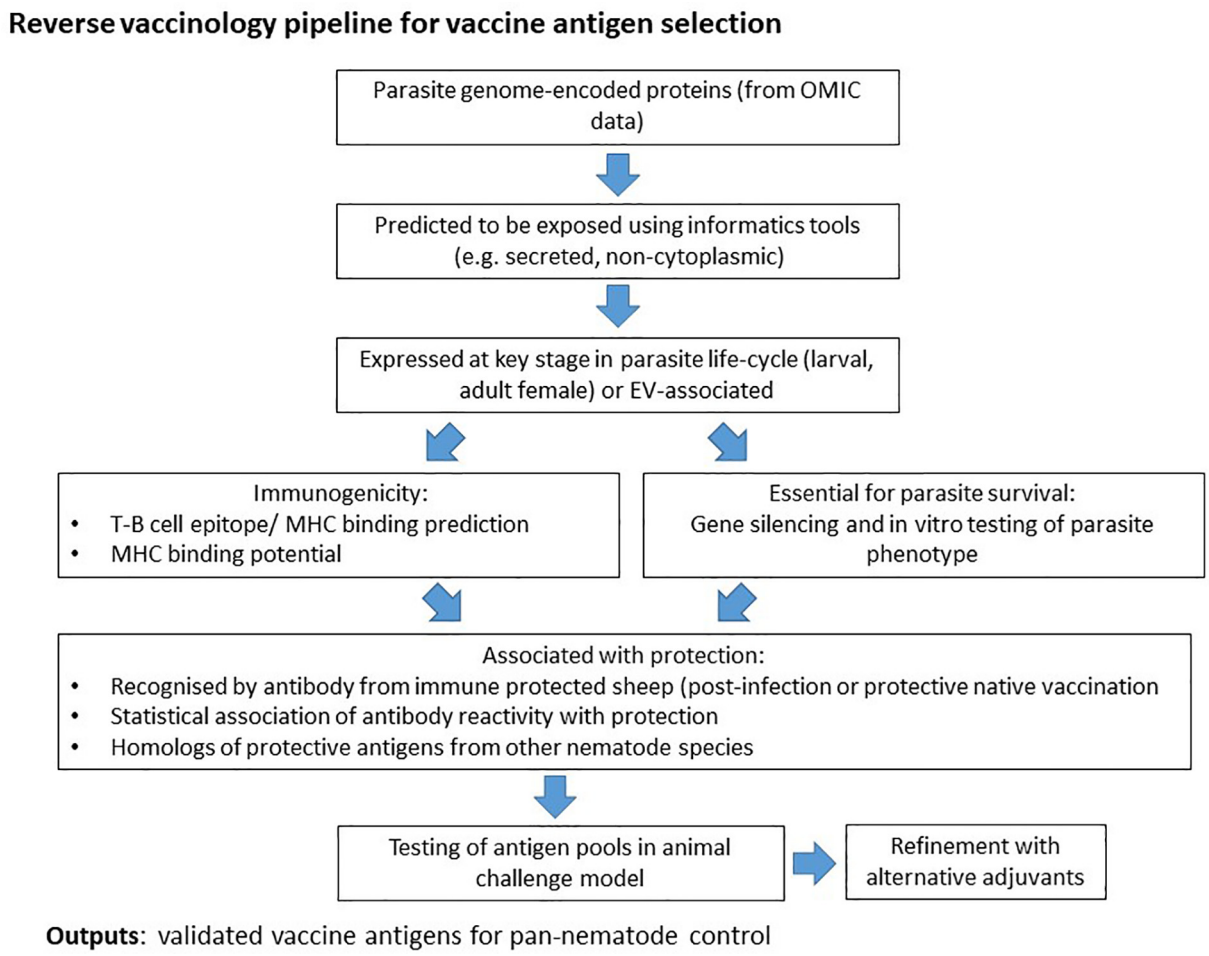

Fig. 3. Reverse vaccinology approach for novel vaccine development for scour worms, involving similar approaches and processes from the parasite genomes utilised by Tabor (2017, 2019) for the tick vaccine. EV, extracellular vesicles; MHC, major histocompatibility complex.

that have greatly enhanced vaccine design for human medicine in recent years (reviewed in Rappuoli et al., 2016) have been:

- development of recombinant mAbs or their antigen binding fragments from cloned B cells derived from immunised (protected) or convalescent (immune) individuals. These can then be used to screen for their neutralising ability against the pathogen (requiring an in vitro assay, see Section 6.5) and to identify the specific epitopes that they bind (see Section 6.2.3);
- high throughput, deep sequencing transcriptomic analyses to analyse the B cell repertoire, understand antibody maturation following infection or immunisation and devise strategies to prime B cell precursors expressing germline-encoded antibodies;
- mapping of conformational epitopes through 3-D analysis of their interactions with the antigen binding fragments of neutralising antibodies;
- new computational approaches to understand the structural and immunological data to design novel vaccines to stimulate specific immunological responses to protective epitopes.

5.2.3. Epitope mapping and peptide arrays

The likely requirement for multiple recombinant antigens to be co-administered to effectively control complex eukaryotic parasitic organisms by vaccination (e.g. see Makepeace et al., 2009; Nisbet et al., 2013) makes the antigen discovery, production and commercialization processes challenging. A potential solution to the latter two issues is to map the most immunogenic epitopes of individual antigens and then to administer these as concatemerised peptides. Using web-based tools, B and T-cell epitopes can be predicted in silico from the protein sequence of potential vaccine antigens (e.g. the Immune Epitope Database and Analysis Resource server at https://www.iedb.org/ and/or the NetCTL server http://www.cbs.dtu.dk/services/NetCTL/). Empirical evidence to underpin the in silico epitope mapping of linear B cell epitopes can be generated using immune or convalescent sera to probe overlapping peptides representing the entire protein either on peptide arrays or in ELISA plate format (e.g. see Dobrut et al., 2018) whereas T cell epitope mapping can be achieved using peptide libraries coupled with ELISpot assays (e.g. see Zhao et al., 2018).

An epitope mapping strategy is being pursued for parasites (e.g. *Trypanosoma cruzi;* Khatoon et al., 2018) where immunogenic epitopes of secretory and membrane proteins were predicted and produced as a synthetic construct, together with molecular docking analysis to optimize the interaction between TLR-2 and TLR-4 receptors and the synthetic vaccine. In parasitic nematodes, Immanuel et al. (2017) described the design, synthesis, and efficacy testing of short peptide epitopes from antigens of filarial nematodes constructed as a "multiple antigen peptide" on an inert lysine core. In jirds, the synthetic vaccine produced high levels of antigen-specific antibody, a cellular response and significant levels of protection (Immanuel et al., 2017).

5.2.4. Immune profiling

Historically, many candidate antigens were differentially identified using serological reagents from small numbers of immune versus susceptible sheep (Emery, 1996; Nisbet et al., 2016) in two-dimensional (2-D) immunoblotting. A more comprehensive immune profiling strategy is summarised in Fig. 3, starting with a comprehensive annotated genome, stage-specific transcriptome, proteome and glycome from the parasite to inform all downstream analyses.

The downstream analyses in such a strategy could employ immunoaffinity chromatography to separate immunoreactive parasite molecules from each parasitic stage, followed by mass spectrometry to identify immunoreactive proteins, genetic variants and glycans within these molecules (as described in Ellis et al., 2014). An additional/alternative approach is to generate high density peptide microarrays, which can now represent up to 2 million peptides on a single chip (Hansen et al., 2017; Østerbye and Buus, 2015). Peptides would be selected using in silico RV approaches to select ORFs encoding membrane or secreted proteins and arrays screened with antibody from multiple immune-protected sheep to precisely define potentially protective linear B cell epitopes (and by extension antigens targeted by CD4+ T cell responses). A similar approach can also be taken to interrogate glycan antibody reactivity in which microarrays consisting of glycans isolated from different parasite life-stages are used for antibody screening (Yang et al., 2018). More focussed, molecule-specific ELISA, peptide and glycan arrays can then be developed to quantify the response to each molecule by immune sheep (using reagents from biobanks, resource flocks or from trials) with ranked FWECs or worm burden data. Sophisticated statistical methods, especially generalized linear mixed modelling, will rank molecules by their influence on resistance to infection.

5.3. Antigen validation by gene silencing

The multiple approaches described above can help select vaccine candidates but validation of these is important for any vaccine development pipeline. This could involve gene knockdown and analysis of resulting phenotypes (e.g. larval arrest, lethality) in vitro. RNAi, where exogenously delivered double-stranded RNA (dsRNA) targets the corresponding mRNA for degradation, has been shown to function very effectively in *C. elegans* by soaking worms in dsRNA, feeding on bacteria expressing dsRNA or microinjection of dsRNA. However, in other nematode species, soaking and feeding are far less effective (Félix, 2008). Using various delivery methods, RNAi has been shown to silence some genes in GIN including *H. contortus* (Geldhof et al., 2006; Kotze and Bagnall, 2006; Blanchard et al., 2018; Menez et al., 2019), *Tel. circumcinta* (Tzelos et al., 2015) and *T. colubriformis* (Issa et al., 2005) (Table 3). By targeting a range of genes, those expressed in the parasite intestine, amphids and excretory system could be silenced, while others were refractory (Samarasinghe et al., 2011). This indicated that for L3s, RNAi is useful for testing the function of genes expressed in sites accessible to the trigger dsRNA but, using current technology, is not suitable for high-throughput functional genomics. In vitro RNAi screening would be the ideal approach and reduce the ethical and cost concerns of in vivo testing. However for some parasite genes, such as those involved in the host-parasite interaction (e.g. mediating host immunosuppression) or required only by developmental stages present within the host, determining effects in vivo, or with improved in vitro culture conditions that mimic the host environment, would be required. Notably, RNAi of the H11 gene in *H. contortus* L3s in vitro prior to infection of sheep resulted in a 57% reduction in FWECs, 40% reduction in worm burden and 64% decrease in aminopeptidase activity (Samarasinghe et al., 2011), supporting use of RNAi for candidate gene identification.

The RNAi pathway is functional in GIN but improvements to the reliability and robustness of silencing and phenotypic or proteomic analysis are needed for this to be applied as a potential vaccine-screening platform (Geldhof et al., 2007; Britton et al., 2016). Stable transfection of nematodes with viral vectors expressing dsRNA to parasite genes is one route to improve on the delivery and persistence of RNAi. Transduction with lentiviral constructs expressing short hairpin RNAs has been shown to induce specific gene silencing and phenotypic effects in *S. mansoni* eggs (Hagen et al., 2014); similar technology is being investigated for the mouse GIN *Nippostrongylus brasiliensis* and may progress RNAi for scour worms (https://gtr.ukri.org/projects?ref=BB%2FS001085%2F1).

An alternative approach being widely applied in mammalian systems and *C. elegans* is CRISPR/Cas9 gene editing. In *C. elegans*, CRISPR/Cas9 gene knockout is highly efficient and specific (Chen et al., 2013). This technology was successfully applied to the human GIN *Strongyloides stercoralis* (Gang et al., 2017; Lok et al., 2017). Efficient delivery of the guide RNA and Cas9 gene/protein are the main hurdles to this being adopted more widely in parasitic nematodes. *Strongyloides* spp. have a free-living cycle and free-living adult female worms can be transfected by microinjection in a similar manner to *C. elegans* (Lok et al., 2017). Effects of CRISPR/Cas9 gene silencing are then examined in the F1 generation following mating. Importantly, the findings of Gang et al. (2017) suggest that in *S. stercoralis*, homozygous gene deletion occurred and was detectable in the F1 generation, without the need for further parasite passage. Although microinjection of individual worms is not a high-throughput approach and not applicable to all nematodes, the use of viral vectors or nanoparticles to deliver CRISPR/Cas9 (reviewed in Lino et al., 2018), combined with improved in vitro culture systems for parasite maintenance (see Section 5.5), may provide a route for robust gene silencing in veterinary GINs.

5.4. Translational silencing

A novel methodology using peptide conjugated phosphorodiamidate morpholino oligomers (PPMOs) allows determination of the phenotype resulting from protein knockdown. PPMOs are non-ionic DNA analogues attached at the 3′-end to a peptide which allows transmembrane movement (Li and Morcos, 2008). Translation of a specific target mRNA in the cell or organism is blocked through Watson/Crick base-pairing of the PPMO with the 5′end of the target mRNA, physically preventing assembly of the ribosome at the initiation codon (Summerton, 1999). This post-transcriptional silencing allows assessment of whether these proteins are essential and potentially good targets for drugs or vaccines. PPMO-mediated translational silencing may be a promising alternative to RNAi and Witola et al. (2016) used this technique to silence phosphoethanolamine methyltransferase expression in adult *H. contortus* in vitro, resulting in a measurable phenotype. Several components are required for successfully carrying out RNAi or PPMO-mediated silencing:

- transcriptomic knowledge to ensure that the target mRNA is expressed in the stage being investigated and that the sequence of the dsRNA or PPMO oligomer is unique to the target gene;
- a method to measure knockdown of the gene or protein (e.g. quantitative (q)PCR, quantitative immunoblotting, qProteomics, enzyme assay);
- a physiologically-relevant method to keep the worms alive in vitro during incubation and downstream phenotypic analysis;
- a thorough assessment of whether the control dsRNA or PPMO (with no-target transcripts in the organism) and any carrier solvents have detrimental effects on the worms in the assay.

Table 3
Summary of gene silencing in *Haemonchus contortus* (*Hco*), *Teladorsagia circumcincta* (*Tci*) and *Trichostrongylus colubriformis* (*Tco*).

Species	Stage	Target gene(s)	Evaluation	Reference
Hco	L3, L4, adult reduced L3 motility	β-tubulin	Reduced mRNA, reduced L4 development	Kotze et al., 2005
	L1-L3	β-tubulin GATA TF Hsp70 Cathepsin L ATPase Superoxide dismutase Paramyosin Collagen Intermediate filament COPII component Calcium binding protein	Reduced mRNA for some targets	Geldhof et al., 2006
	L3	H11 ASP-1 β-tubulin GTPcyclohydrolase Aquaporin *apq-2* Helicase *phi-10* Transcription factor *ceh-6* Chloride channel *exc-4* Ribosomal genes	Reduced mRNA for some targets Reduced in vivo survival H11 RNAi	Samarasinghe et al., 2011
	L1, L3, adult	Ubiquitin β-tubulin paramyosin Tropomyosin ATPase *vha-19* Mechanoreceptor *noah-1* *mitr-1* galactopyranose mutase *glf-1* *pat-12*	Reduced mRNA for some targets Reduced viability following RNAi feeding	Zawadzki et al., 2012
	L2	*unc-38* *unc-63*	Unco-ordinated motility	Blanchard et al., 2018
		acetylcholine receptor *acr-8*	Reduced levamisole sensitivity	Blanchard et al., 2018
		nuclear hormone receptor *nhr-8*	Increased ivermectin sensitivity	Menez et al., 2019
	Adult	[a]PMT1&2 Phosphoethanolamine N-methyltransferase	Reduced protein& reduced viability	Witola et al., 2016
Tci	L3	ASP	Reduced mRNA	Tzelos et al., 2015
Tco	L1	Tropomyosin Ubiquitin	Delayed development to L3	Issa et al., 2005

[a] All studies carried out by RNAi except this study by Witola et al. which used morpholino oligomers TF, GATA transcription factor; hsp70, heat shock protein 70; ASP-1, activation-associated secretory protein 1; pat, paralysed arrest at two-fold; unc, unco-ordinated.

This final point is particularly pertinent to the use of PPMOs with scour worms as PPMOs require to be at relatively high concentrations in the culture medium (90 μM for work published by Witola et al. (2016)). Preliminary work with xL3s *Tel. circumcincta* has suggested that micromolar concentrations of a control and a gene-specific PPMO are both toxic to this stage of the worm (Nisbet et al., unpublished data).

5.5. In vitro culture

Improved in vitro culture of GIN should enable better delivery of RNA or DNA for gene silencing or screening for effective neutralizing antibodies to establish vaccine screening platforms. Ideally, effects on worm motility, moulting or survival could be detected using automated systems over longer timespans than in current drug screens. Previous work reported in vitro development of *H. contortus* infective larvae to reproducing adult worms using abomasal extracts (Stringfellow, 1986). More recent studies have used cell co-culture to achieve improved development of GIN. For example, co-culture with Caco2 cells, a human gut epithelial cell line previously used for *T. spiralis* culture (Gagliardo et al., 2002), promotes development of *H. contortus* L3s to L4 stage (Britton et al., 2016), while the human colonic cell line HT-29 supports development of *T. colubriformis* L3 (Andronicos et al., 2012).

An "in vitro direct challenge" (IVDC) method was developed to examine the initial immune exclusion/prompt rejection mechanism operating in the abomasa of sheep immune to *Tel. circumcincta* (Jackson et al., 2004) or *H. contortus* (Kemp et al., 2009). Abomasal tissue explants were maintained in buffer in a high oxygen concentration, then challenged with xL3s. xL3s that penetrated gastric glands or had been excluded/rejected were then recovered. While this technique is unlikely to maintain parasitic stages of the nematodes for periods longer than a few hours, the methodology was adapted by Nisbet et al. (2009) to test whether antibodies to *Tel. circumcincta* vaccine candidate Tci-SAA-1 were able to inhibit the larvae from establishing in abomasal explants from helminth-naïve lambs.

Recent successful development of intestinal organoids (Sato et al., 2011; Powell & Behnke, 2017; Hamilton et al., 2018) provides another in vitro technology that may support GIN development for target screening. Intestinal organoids (mini-guts) are derived from single stem cells isolated from the intestinal crypts. These LGR5^{+} stem cells divide and differentiate in vitro under suitable conditions (with growth factors) to produce all the cells characteristic of gut epithelium including goblet cells, enterocytes, Paneth cells,

tuft cells and enteroendocrine cells. Similar organoids could be generated from the gastric epithelium for the abomasal parasites. Organoids can be serially passaged long-term and may provide a more physiological epithelium culture system in which parasite larvae may be maintained and host-parasite interactions examined. Duque-Correa et al. (2020) are using tissue organoids to study host-parasite interactions and parasite larval development, particularly for *Trichuris muris*. GIN infective larvae are larger than *T. muris*, making their introduction into 3-D organoid structures more difficult. However, once established, 3-D organoids can also be cultured as a 2-D epithelial layer, which would enable greater access and penetration of parasites. This potentially provides a physiological system to develop and maintain veterinary nematodes for vaccine candidate screening. Alternatively, ILC2 cells involved in Th2 responses in gut (helminths) and lungs (Neill et al., 2010) may provide an additional means to screen for the developmental intricacies of host-parasite reactivity.

5.6. microRNAs and extracellular vesicles

In addition to RNAi-mediated gene silencing, nematodes express small regulatory microRNAs (miRNAs) that silence genes post-transcriptionally. In *C. elegans*, some of these miRNAs play essential roles in regulating development and responses to environmental stress (reviewed in Britton et al., 2014). Some of the miRNAs present in *C. elegans* are conserved in veterinary GIN (Winter et al., 2012; Gu et al., 2017), and likely act to regulate development and metabolism. While miRNAs may themselves be targets for therapeutic intervention (Britton et al., 2014), determining the pathways they regulate as parasites develop within the host has the potential to identify novel control targets (Marks et al., 2019).

Further to regulating endogenous genes within the parasite, miRNAs are also released from GIN including *Tel. circumcincta*, *H. contortus* (Gu et al., 2017) and *Heligmosomoides polygyrus* (Buck et al., 2014). miRNAs have been sequenced from in vitro ES products, both in the supernatant and in extracellular vesicles (EV) isolated from ES by differential ultracentrifugation (Buck et al., 2014; Gu et al., 2017). Parasite-derived miRNAs have also been identified in host plasma following filarial infections, suggesting that they are also released in vivo (Tritten et al., 2014). Importantly, secreted miRNAs present in EV have been shown to modulate host immune response genes, suggesting that they may function at the host-parasite interface to promote parasite survival and/or suppress immune-mediated damage (Buck et al., 2014). Recently, the proteome of EVs released from *Tel. circumcincta* has been characterised and includes a number of putative immunomodulatory proteins which are targeted by both IgA and IgG antibodies from infected sheep (Tzelos et al., 2016). Targeting EV through vaccination may therefore neutralize immunoregulatory effects and enhance immunity. Indeed, recent vaccination studies in mice using 1.5–3 μg of EVs prepared from ES products of cultured adult *H. polygyrus* or *T. muris* nematodes and inoculated without adjuvant, reduced worm burden by approximately 50%, by inducing antibodies that promote uptake and processing by antigen presenting cells (APC) (Coakley et al., 2017; Shears et al., 2018). EVs from *T. muris* contained approximately 125 proteins, such that through their uptake by APC, EVs may provide a novel vaccine formulation (Shears et al., 2018).

6. Field integration and evaluation of vaccines

Vaccines against sheep nematodes are envisaged as a component of IPMs. Vaccination suppresses pasture contamination with worm eggs, with subsequent reductions in worm burdens. Fortunately, ecological research over many years has provided a detailed understanding of seasonal patterns for the major species in the major "type" environments (e.g. O'Connor et al., 2006), which will serve as the basis for computer simulation modelling of optimal vaccination schedules for various locations, followed by in-field validation.

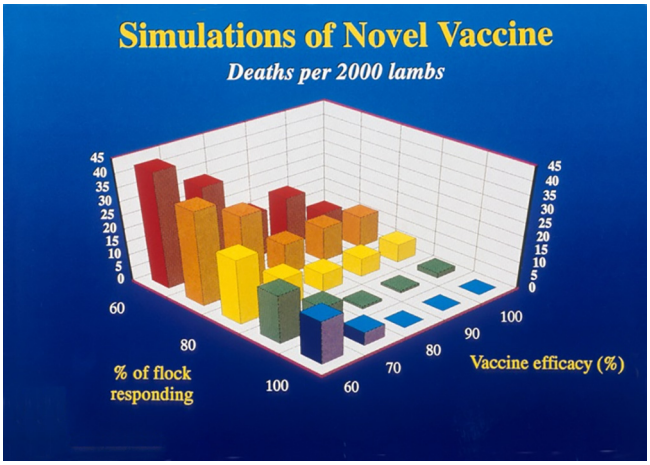

Fig. 4. Simulations of vaccines with varying levels of efficacy and differing proportions of host responders on the numbers of deaths from scour worms in a flock. Deaths occur in lambs when worm numbers reach 2500 (modified from Barnes et al., 1995).

One of the key questions remains, "How much protection is sufficient for my vaccine?" Several models are available to test hypotheses (Dobson et al., 2011; de Cisneros et al., 2014; Laurenson and Kahn, 2018). Previous modelling studies (exemplified in Fig. 4) indicated that prevention of deaths in weaners with concomitant reductions in pasture L3 could be achieved with vaccines which gave 70–80% protection (reduced FWECs) in ~80% of vaccinated animals. Death was presumed to occur with *Trichostrongylus* worm burdens of >2500 (Fig. 4). These simulations should be extended to incorporate the various vaccine efficacies in concert with additional interventions normally associated with IPMs – for example the interactions between vaccine efficacy and nutrition (or strategic anthelmintic use) to give adequate protection. This will need empirical data from pen and field studies in which vaccines of known efficacy are tested, even if their protective capacity is lower than the previously determined minimum thresholds. At this point, since current IPM utilise seasonal pasture L3 levels from field data that is more than 30 years old, no modelling on the effects of vaccination on pasture L3s has been done.

7. Conclusions

The development, formulation and delivery of a successful GIN vaccine requires an understanding of three major components (see Fig. 5):

(i) the protective responses to be induced by the vaccine;
(ii) the most appropriate suite of molecules that induce effective immunity;
(iii) the ability of different vaccine formulations (+/− adjuvants) to induce potential protective immune responses against GINs in sheep mucosa.

To address (i): Th2 responses are protective against scour worms in sheep (see Section 2). However, the main targets of this immune response in the worms are poorly understood at this stage and methods for inducing the appropriate parts of the immune sys-

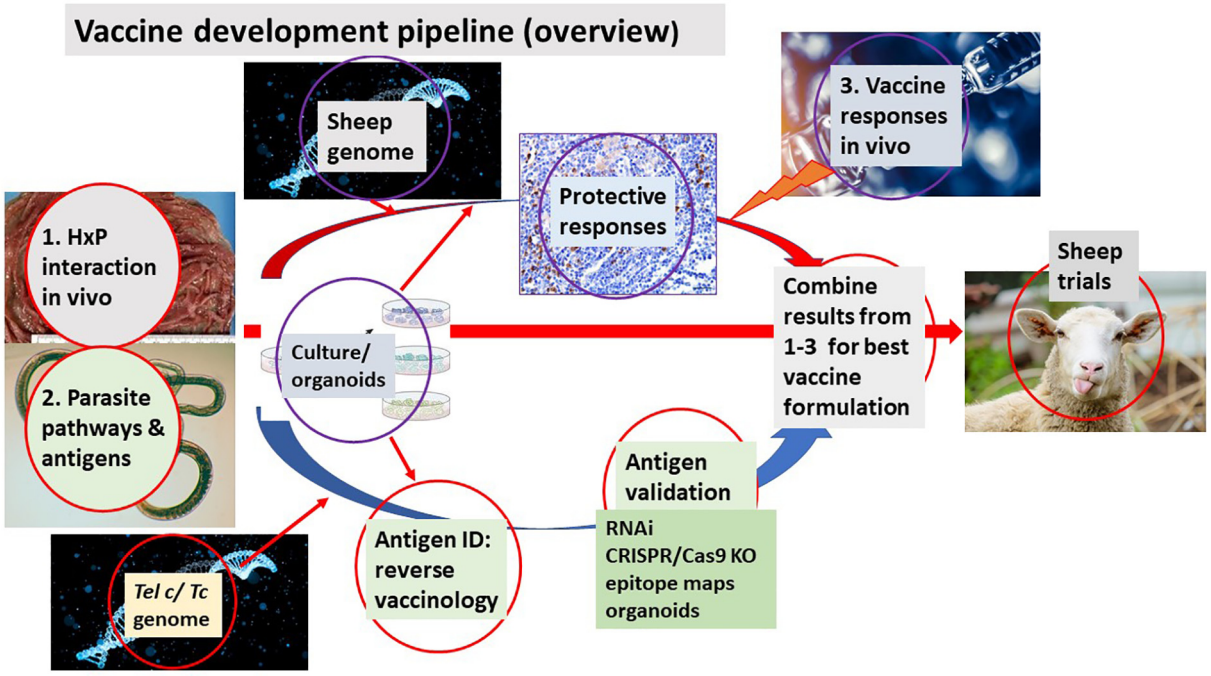

Fig. 5. A generalised overview of the integrated process pipeline for scour worm vaccines. The three areas of (1) HxP (host by parasite) interaction; (2) antigen discovery and validation and (3) vaccine formulation and performance come together to formulate verified protective antigens (2) into the most effective vaccine preparation (3) to deliver and elicit the protective response(s) identified from (1). *Tel c/Tc*, *Teladorsagia circumcincta*/ *Trichostrongylus colubriformis*.

tem through vaccination are also lacking. This needs a systematic, dynamic investigation of the host-parasite interaction in vivo during the 3–6 weeks taken to develop immunity and worm patency (Fig. 5).

To address (ii): many vaccine "candidate" antigens have been identified, purified and trialled as native or recombinant vaccines against *Tel. circumcincta* and *T. colubriformis*; there is no shortage of potential antigens. However, with a long-term approach similar to the successful Cattle Tick vaccine using RV from an annotated genome, a new opportunity is available for scour worms. Vaccines for scour worms may require several components to be developed through a RV approach and rationally integrated into the vaccine development program (i-iii above; see Section 5). Once these antigen/ pathway targets in the worms have been identified (i), a screening method is needed to allow the identification of the best targets from potentially large numbers of candidates at the start of the process. Currently this would rely on screening in the animals but the development of novel methods to do this in the laboratory would be faster, reduce potential ethical limitations and be more cost-effective, and a set of potential techniques to address this is presented in the review (see Section 5). A prerequisite is an appreciation of what has already been attempted for these and other species and what current and emerging technologies might be employed to produce and evaluate novel vaccines. These include new 'omics' technologies as well as antigen validation by methods such as gene knock-out or silencing. Each of these aspects is addressed in the review and, although there is substantial knowledge in most of these areas, fundamental gaps exist in knowledge, available resources and technologies (above) to allow a fully functional RV approach.

To address (iii): we know how to induce high levels of "protective" antibody in sheep using a range of "immunostimulants" or adjuvants (see Section 3). What is not known is exactly how the host-parasite interaction over several weeks manages to generate the protective immunity to prevent parasitism. In the absence of definitive information of the (stage-specific) protective response or its induction in ruminants, most vaccines containing purified or recombinant antigens have used an empirical approach. We do not know how to induce mucosal immunity with a vaccine. While we recommend the kinetic analysis of the host-parasite interaction to provide insights into (i), a similar approach is needed to provide information on the effects of vaccine formulations on mucosal immunity so that we can formulate any potential antigens into a vaccine that induces the responses identified as protective by (i); thus integrating the three approaches needed for optimising vaccine success. A meta-analysis of the inductive capabilities of current vaccine adjuvants for ruminants may assist here.

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