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# Veterinary Immunology Committee Toolkit Workshop 2010: Progress and plans

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### ABSTRACT

The 3rd Veterinary Immunology Committee (VIC) Toolkit Workshop took place at the 9th International Veterinary Immunology Symposium (IVIS) in Tokyo, Japan on 18th August 2010. The Workshop built on previous Toolkit Workshops and covered various aspects of reagent development, commercialization and provision to the veterinary immunology research community. The emphasis was on open communication about current progress and future plans to avoid duplication of effort and to update priorities for reagent development. There were presentations on the major reagent development and networking projects such as the BBSRC/RERAD Immunological Toolbox (2004–2009), US Veterinary Immune Reagent Network (VIRN 2006–2010) that has just received renewal funding for 2010–2014, and EU Network for Animal Diseases Infectiology Research Facilities project (NADIR 2009–2013). There were also presentations and discussions on the use of reagents for assay development, particularly multiplexing, and how these new technologies will underpin basic research developments. Mechanisms for improved information exchange, especially through websites with VIC playing a central role, were identified.

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## 1. Introduction

The Veterinary Immunology Committee (VIC) is one of six Committees that operate under the auspices of the International Union of Immunological Societies (IUIS; <http://www.iuisonline.org/pages/committ.htm>). The current Chair of VIC is Wayne Hein, who oversees a number of Subcommittees and Working Groups that address different areas of veterinary immunology, one of these subgroups being VIC Toolkit Committee. The VIC Toolkit Committee aims to provide a global network for information exchange to accelerate reagent development, facilitate reagent exchange and find means of protecting valuable and irreplaceable reagents that are in danger of becoming 'orphaned'. This is achieved in a number of ways, pri-

marily through electronic communication given the global distribution of the members. However, once every three years the International Veterinary Immunology Symposium (IVIS) brings together the veterinary immunology community at one meeting and provides an excellent opportunity for interaction and discussion. The seeds for the VIC Toolkit were sown at a Workshop to discuss veterinary immunological reagents held during the 6th IVIS in Uppsala, Sweden in 2001 (Entrican, 2002). The inaugural VIC Toolkit Workshop took place at the 7th IVIS in Quebec City, Canada in 2004 and the second at the 8th IVIS in Ouro Preto in 2007. The full details of the structure of VIC Toolkit Committee and its objectives can be found in the report on the meeting in Ouro Preto (Entrican et al., 2009). Here we report on the third VIC Toolkit Workshop held at the 9th IVIS in Tokyo, Japan in August 2010. The Workshop was structured to provide updates on the major reagent development initiatives globally as well as covering more specific aspects of reagent applications and evaluations.

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## 2. The BBSRC/RERAD Immunological Toolbox

Gary Entrican (Moredun, UK) began the session by presenting a final report and describing the outputs of the BBSRC/RERAD Immunological Toolbox (2004–2009). The Immunological Toolbox was a three-centre Consortium project awarded to the Institute for Animal Health (IAH), the Animal Health Trust (AHT) and the Moredun Research Institute (MRI) to develop immunological reagents for five veterinary species (cattle, sheep, pigs, horses and chickens). The targets for each species were identified according to gaps in existing capability and, whenever known, taking into account what was being done in those species at other organizations (most notably by the United States Veterinary Immune Reagent Network [US VIRN], see below). In addition to developing reagents and assays, the objectives of the Immunological Toolbox also included reagent provision and knowledge exchange through publications, presentations and the development of a dedicated website and database. The project was initially coordinated by Jim Kaufman at IAH (2004–2007) then latterly by Gary Entrican at MRI (2007–2009). Among the achievements of the Toolbox were the cloning and expression of pig  $\beta$ 2-microglobulin and MHC class I molecules, expression of more than 20 cytokines and chemokines of cattle, sheep, horse and chicken origin, development of quantitative PCR assays for over 70 cytokines, development of PCR detection of equine MHC Class I and production of polyclonal antisera and monoclonal antibodies (mAb) to cytokines, chemokines and cell-surface proteins. Details can be found on the Immunological Toolbox website (<http://www.immunologicaltoolbox.co.uk/>) and wherever possible these reagents have been commercialized through AbD Serotec (<http://www.abdserotec.com/>). The Toolbox outputs also included 22 peer-reviewed publications in scientific journals and 3 book chapters describing reagent development and/or reagent characterisation.

Several lessons were learned during the course of the Toolbox project, such as the added value of working within a Consortium sharing skills, knowledge and resources. Reagent development did not always go to plan, even when approaches that had successfully worked for other targets, or the same target in other species, were taken. The failure of the Immunological Toolbox to produce mAb to bovine IL-2 despite multiple attempts and several approaches was a good example of how unpredictable and frustrating reagent development can be. Even when new mAb are produced, the characterisation and validation process, particularly when ELISA development is involved, is very time consuming. The mAb produced to ovine CXCL8 by the Toolbox are a case in point and an ELISA will be available once it is fully optimized and specificity is confirmed.

One of the things that distinguished the Toolbox from US VIRN was the coordinated reagent development for cattle and sheep. These two species are very closely related phylogenetically, hence the likelihood of reagent cross-reactivity is very high. Examples are the mAb-based sandwich ELISAs that detect both cattle and sheep IL-4, IL-10, IL-12 and IFN- $\gamma$  (Hope et al., 2002, 2005; Wattedegera et al., 2004, 2008). However, this is not always the case and cross-reactivity cannot be assumed. Previous work had shown that a sand-

wich ELISA based on mAb produced against ovine GM-CSF did not detect bovine GM-CSF. Similarly, during the course of the Toolbox project a sandwich ELISA based on mAb produced against bovine TNF- $\alpha$  did not detect ovine TNF- $\alpha$ . By cross-checking the mAbs against recombinant proteins of each species and conducting intracellular cytokine staining and flow cytometry on transfected cells it was discovered in each of these cases that one mAb of the ELISA pair did not recognize the orthologous cytokine (Entrican et al., 1996; Kwong et al., 2010). Further funding for the Immunological Toolbox was sought towards the end of 2009 but this was unsuccessful. The future for UK veterinary reagent development may be via smaller projects, possibly supported in part by industry.

## 3. NADIR

Although the Immunological Toolbox has come to an end, there is a Consortium-based research and networking project that was recently established within the European Union (EU), the Network for Animal Diseases Infectiology Research Facilities project (NADIR), funded by the EU Seventh Framework Programme. The strategic aim of NADIR is to realize the EU potential in animal infectiology by bringing together experimental laboratories to optimize their studies, improve tools, share reagents and achieve economies of scale ([http://www.nadir-project.eu/nadir\\_project/](http://www.nadir-project.eu/nadir_project/)). There are 15 organizations across Europe that participate in NADIR, all with facilities to conduct research into infectious diseases of farmed livestock. The project was launched in France in May 2009 and is funded until 2012. NADIR has a number of networking and research activities that can be found at [http://www.nadir-project.eu/nadir\\_project/layout/set/print/the\\_project](http://www.nadir-project.eu/nadir_project/layout/set/print/the_project). Of interest to veterinary immunologists within the EU may be the NADIR Transnational Access Programme that provides funds for those not within the NADIR Consortium to support short-term visits to laboratories within NADIR. Details on how to apply for these visits can be found at [http://www.nadir-project.eu/nadir\\_project/call\\_for\\_access](http://www.nadir-project.eu/nadir_project/call_for_access).

## 4. US VIRN

Joan Lunney (USDA ARS BARC, Beltsville, USA) provided an update on US VIRN before describing progress on the Swine Toolkit component of the network. US VIRN is a multi-centre Consortium project funded by USDA to develop immunological reagents for cattle, pigs, horses, chickens and fish (trout and catfish). The dedicated website can be found at <http://www.vetimm.org>. US VIRN and the Immunological Toolbox have worked closely together and complement each other as been described previously (Entrican et al., 2009). US VIRN was funded for four years from 2006; the four year renewal application was recently approved. One major difference between the research funding in the US and UK is the mechanism for involving a commercial partner within a government-funded project. For the Research Councils within the UK the industrial partner would be expected to contribute financially

to the project whereas in US VIRN, the commercial partner Kingfisher Biotech (<http://www.kingfisherbiotech.com/>) is paid from the project to express proteins to contribute to the effort. The overall aim of US VIRN is to systematically address the immunological reagent gap for the veterinary immunology research community. Kingfisher is intrinsically part of delivering that aim. The specific goals of US VIRN are to clone, sequence and express immune proteins (cytokines/chemokines) either in *Pichia pastoris* (yeast) through Kingfisher or using mammalian or bacterial expression systems at species laboratories; test bioactivity of these immune proteins; produce specific mAb to these proteins; use mAb to develop assays such as ELISAs or Fluorescent microbead immunoassays (FMIA) to quantitate immune proteins; and produce mAb to cell surface immune markers such as T cell receptors (TCRs) and cell surface differentiation (CD) molecules.

Experience within US VIRN shows that the yeast expression system does not seem to work as well for fish or chicken proteins as it does for the mammalian proteins. Hence, for fish and some chicken proteins, *Escherichia coli* is the expression system of choice; mammalian expression systems are also being tested despite limited protein yields. As part of the US VIRN renewal a coordinated approach for catfish and trout gene cloning and expression is being used. Recent progress within US VIRN for these species has been the production of mAb to catfish recombinant IgL $\alpha$  to phenotype B cell subsets in peripheral blood and also to separate cells for functional assays (Edholm et al., 2010). In chicken, a number of genes have been cloned and sequenced with the ultimate aim of producing mAb. These include IL-1 $\beta$ , IL-2, IL-4, IL-10, IL-12p35, IL-12p40, IL-15, IL-16, IL-17D, IFN- $\gamma$ , LITAF, NK-lysin, TNFSF15, CCL4, CCL20 and CXCL1. The chicken cell-surface proteins that have been cloned include CD80, CD83, CD86, CXCR4 and the IL-2R $\gamma$  chain (see <http://www.umass.edu/vetimm/poultry/index.html>).

#### 4.1. US VIRN Swine Toolkit

Joan Lunney described progress within the Swine Toolkit using a flow chart to show the current position of the target molecules at the various stages of cloning cDNA through to protein expression, purification and bioactivity, immunization of mice, performing fusions, characterisation of mAb and development of assays. This chart is accessible on the US VIRN swine website (<http://www.umass.edu/vetimm/swine/index.html>). The porcine chemokines and cytokines that are currently cloned and are targets for bioassay, mAb development and/or FMIA development include CCL2, CCL3L1, CCL4, CCL5, CCL20, CCL25, CCL28, CXCL9, CXCL10, CXCL11, IFN- $\alpha$ 1, IFN- $\alpha$ 6, IFN- $\beta$ , IFN- $\gamma$ , TNF- $\alpha$ , IL-6, IL-7, IL-13, IL-15, IL-17A, IL-17F and IL-22, with IL-9, IL-21 and IL-23A as future targets for cloning (Boyd et al., 2010). Cell-surface targets include IgG1, IgG2, IgG3, IgG5, TCR $\alpha$ , TCR $\beta$ , IL-4R $\alpha$ , IL-13R $\alpha$ , IL-17R $\alpha$ , IFN $\alpha$ , CXCR3, CXCR5, CD1c, CD19, CD45RO, NKp30, NKp44, and NKp46 (NCR1/2/3 respectively). Of these targets, a panel of mAb has recently been made to porcine CCL2 and (unfortunately) shown not to cross-react with either equine or bovine CCL2.

As previously mentioned, assay development is a feature of future US VIRN efforts. These assays are the ultimate application of reagent projects since they facilitate hypothesis-driven research into disease pathogenesis and underpin vaccine design. There are several commercial mAb to porcine cytokines/chemokines that have recently been incorporated into a novel FMIA to simultaneously measure IFN- $\alpha$ , IFN- $\gamma$  TNF- $\alpha$ , IL-8 (CXCL8), IL-1 $\beta$ , IL-4, IL-10 and IL-12 during immune responses to porcine reproductive and respiratory syndrome virus (PRRSV) (Lawson et al., 2010). Additional multiplex assays are being planned for other targets as sets of mAb become available, e.g., for CCL2.

#### 4.2. US VIRN Equine Toolkit

Bettina Wagner (Cornell, USA), Horse Species Coordinator for US VIRN, began by re-iterating the importance of two-way dialogue for reagent development. Users with particular needs that are not yet met should contact the relevant species coordinator within US VIRN for consultation (<http://www.vetimm.org>). As for the other species, the equine targets include expression of recombinant cytokines, chemokines and cell-surface molecules, production of mAb to the recombinant proteins and development of assays with the reagents produced. Recent successes include the production of mAb to equine CD14 (Kabitha et al., 2010), FMIA for simultaneous measurement of equine IFN- $\alpha$ , IL-4 and IL-10 (Wagner and Freer, 2009) and triple intracellular cytokine staining to define T cell subsets (Wagner et al., 2010). The ongoing cytokine/chemokine targets are equine CCL3, CCL5, CCL11, IL-5, IL-6, and IL-17A. Cell-surface targets are CD19, CD23, CD25, CD28, CD40, TCRs and Fc $\epsilon$ R1. As part of the US VIRN renewal a broader approach is being used for simultaneously cloning all three mammalian orthologues (equine, swine, bovine) and testing mAb for cross reactions between species. In addition anti-bovine mAb are shared with UK Toolbox partners for tests of reactivity with sheep orthologues.

The Equine Toolkit has exploited a fusion approach for protein expression using cytokine and immunoglobulin sequences to provide identification and purification tags as well as immune boosting potential. Yeast (*Pichia*) and mammalian (Chinese hamster ovary [CHO] cells) expression systems have been used in different cases since some molecules can be produced more readily in one system than the other. The two systems can also complement each other in screening hybridomas at an early stage. Much effort can be wasted on mAb that only recognize the immunogen, namely the recombinant protein, and not necessarily the native protein, and are therefore useless in bioassays and detection systems. Anti-equine IL-2 mAb produced against yeast-expressed protein were screened at an early stage against mammalian-expressed equine IL-2 in CHO cells by flow cytometry, hence enhancing the likelihood of selecting the most useful hybridomas. Similarly, CHO cells transfected with cDNA encoding equine CD23 were used to definitively confirm the failure of an anti-human CD23 mAb to cross-react with the equine orthologue and thus inform on priorities for reagent development. A similar approach had been used for ovine and bovine cytokines within the Immunological Toolbox. It was agreed that transfected CHO

cells are an excellent positive control for testing cross-reactive mAb.

### 5. Reagent application and evaluation

The purpose of developing reagents for veterinary immunology is to inform on host immunity and disease pathogenesis, thereby support the design of safe and effective disease control and vaccination strategies. The workshop included two short presentations that both involved the use and/or evaluation of species-cross mAb. The first was given by Wilhelm Gerner (Vienna, Austria) on the detection of Foxp3 expression in bovine peripheral blood mononuclear cells (PBMC). At the outset he stated that a mAb had recently been produced to bovine Foxp3 (Seo et al., 2009). His experiments used a commercially available anti mouse/rat Foxp3 mAb (FJK-16s, eBioscience; <http://ebioscience.com/>) that had previously been confirmed to detect porcine Foxp3 using HEK293T cells transfected with cDNA encoding a sequence of porcine Foxp3 corresponding to the predicted epitope recognized by the mAb (Bolzer et al., 2009). This sequence is identical in porcine and bovine Foxp3 which therefore predicts cross-reactivity of the mAb with cattle. The mAb was used in flow cytometric analysis of bovine PBMC and spleen cells following permeabilisation. Multiple controls were used to confirm specificity, including the use of a dead-cell marker, competition-labelling with non-fluorescent FJK-16s and non-specific isotype-matched controls. All Foxp3-positive cells expressed CD3 and the majority, but not all, were CD4+ve. The largest percentage of Foxp3+ve cells was found in cattle lymph nodes (around 5%), which was 10–20 fold greater than the percentage in blood and spleen (less than 0.5%). The results of this work have recently been published in full (Gerner et al., 2010).

Andrey Lage (Minas Gerais, Brazil) gave a presentation on the use of flow cytometry to identify commercially available anti-human and anti-mouse cytokine mAb against that could cross-react with ruminants. Nine mouse anti-human mAb (against IL-1, IL5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17 and TNF- $\alpha$ ) and two rat anti-mouse mAb (against IL-10 and TNF- $\alpha$ ) were tested on PBMC from cattle, sheep and goats activated in the presence of PMA/ionomycin and cytokine secretion blocked with brefeldin. There was potential cross-reactivity with cattle, goats and sheep for some of the mAb. There was a discussion regarding specificity and there was agreement that competition-labelling with non-fluorescent mAbs and positive controls, using transfected cells as described earlier, is necessary to give the most definitive affirmation of cross reactivity.

### 6. Panel discussion, future directions and closing remarks

It was clear from the VIC Toolkit workshop discussion that despite recent progress, the veterinary immunology research community still has many unmet reagent needs. Some reagents that are provided on a collaborative basis are yet to become commercially available and other reagents

appear to have been lost from the market (porcine IL-6 mAb and an anti-bovine TNF- $\alpha$  ELISA kit for example). Reagent transfer is not always straightforward since it involves issues over protection of intellectual property (IP), royalty streams, restriction of use, importation permits, exclusivity, patents and licences. These issues can also impact on the exchange of reagents through material transfer agreements (MTAs). All of these processes are surmountable (and often necessary) and hence need to be dealt with on a case-by-case basis for commercialization and also adoption of orphan reagents. Additional support for veterinary immune reagent repositories would clearly assist these efforts.

There was a question of making reagents available as a condition of publication. Such conditions cannot be imposed for the reasons described above – existing licences, IP issues, and importation restrictions can prevent distribution of certain materials. Even without these restrictions it was noted that recipient organizations sometimes make demands or disagree with the conditions of MTA after requesting reagents. Depending on the degree of alteration required, this can stall or even abort the transfer process.

Funding issues were also discussed and the involvement of industrial partners with research projects. As described earlier, different funding organizations have different views and requirements on academic-industrial partnerships. Some concerns were raised over terms and conditions, such as exclusivity, of certain commercial partnerships. Additionally, currently available commercial reagents may not remain on the market. If a company is taken over or changes its marketing strategy, reagents could be lost to the research community if, for example, they were viewed as non-profitable.

There is also an unmet need for information on the techniques and experimental procedures for using reagents and affirming their reactivity. Current websites list reagent availability but limited protocols on how they should be used. Scientific papers do not usually contain sufficient detail and certainly not tips on usage. One method might be to make better use of the Vetimm global discussion forum ([vetimm@vasci.umass.edu](mailto:vetimm@vasci.umass.edu)) and specific websites (such as US VIRN [www.vetimm.org](http://www.vetimm.org)) with regular updates. This includes the addition of negative data when protocols or reagents do not work. There was agreement that feedback should also be given to reagent providers if well-controlled negative data are generated. This includes commercial companies since it will prevent others wasting time and money on reagents that will not work. One example used was the assumption that recombinant cytokines will be biologically active, which is not always the case.

There was a discussion on VIC Toolkit Committee and how it delivers on the key objectives of importance to VIC and ultimately to IUIS. There is clearly scope for better communication and activity. As the IUIS VIC establishes a separate VIC webpage it was recommended that the VIC Toolkit Committee should have a webpage accessed via that updated VIC webpage within IUIS website. This will help promote VIC as an entity and, with hyperlinks to other sites, act as part of an integrated network for veterinary immunologists worldwide.

## Conflicts of interest

GE has developed veterinary immunological reagents that are commercially available through AbD Serotec. Royalties from sales are paid to Moredun Research Institute. JKL has no commercial interests. The US VIRN will issue non-exclusive licenses for commercialization of any reagent it developed.

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