



## Identification of cross-reacting T-cell epitopes in structural and non-structural proteins of swine and pandemic H1N1 influenza A virus strains in pigs

**Baratelli, Massimiliano; Pedersen, Lasse Eggert; Trebbien, Ramona; Larsen, Lars Erik; Jungersen, Gregers; Blanco, Esther; Nielsen, Jens; Montoya, Maria**

*Published in:*

Journal of General Virology

*Link to article, DOI:*

[10.1099/jgv.0.000748](https://doi.org/10.1099/jgv.0.000748)

*Publication date:*

2017

*Document Version*

Publisher's PDF, also known as Version of record

[Link back to DTU Orbit](#)

*Citation (APA):*

Baratelli, M., Pedersen, L. E., Trebbien, R., Larsen, L. E., Jungersen, G., Blanco, E., ... Montoya, M. (2017). Identification of cross-reacting T-cell epitopes in structural and non-structural proteins of swine and pandemic H1N1 influenza A virus strains in pigs. *Journal of General Virology*, 98(5), 895-899. DOI: 10.1099/jgv.0.000748

---

### General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

# Identification of cross-reacting T-cell epitopes in structural and non-structural proteins of swine and pandemic H1N1 influenza A virus strains in pigs

Massimiliano Baratelli,<sup>1</sup> † Lasse Eggers Pedersen,<sup>2</sup> † Ramona Trebbien,<sup>2,3</sup> Lars Erik Larsen,<sup>2</sup> Gregers Jungersen,<sup>2</sup> Esther Blanco,<sup>4</sup> Jens Nielsen<sup>2,3</sup> and Maria Montoya<sup>1,5,\*</sup>

## Abstract

Heterologous protection against swine influenza viruses (SwIVs) of different lineages is an important concern for the pig industry. Cross-protection between 'avian-like' H1N1 and 2009 pandemic H1N1 lineages has been observed previously, indicating the involvement of cross-reacting T-cells. Here, reverse vaccinology was applied to identify cross-reacting MHC class I T-cell epitopes from two different SwIV H1 lineages in pigs. *In silico* prediction followed by *in vitro* and *in vivo* testing was used to identify SLA-1\*0702 T-cell epitopes in heterologous SwIV-infected pigs. Following viral infection, tetramer specific T-cell populations were identified. The majority of the identified T-cell epitopes were conserved between the examined lineages, suggesting that targeting cross-reactive T-cell epitopes could be used to improve vaccines against SwIV in SLA-1\*0702-positive pigs.

Swine influenza virus (SwIV) is considered to be an important pathogen in pig herds. Different subtypes and lineages are circulating in pigs [1] and the involvement of both arms of the immune system is considered to be necessary for an effective immune response [2]. Although commercially available vaccines were designed to confer homologous protection, the control of heterologous infections is of high importance for controlling influenza in pig herds. The identification of protective cross-reacting antigens thus remains an open challenge.

Some previous studies have shown that infection with SwIVs of European 'avian-like' H1N1 lineage can induce complete protection against the influenza A virus of the 2009 pandemic H1N1 lineage [3, 4]. Such cross-protection was conferred in the absence of cross-reactive antibodies capable of inhibiting hemagglutination or virus neutralization, suggesting that other factors, like cross-reacting T-cell responses, might have been involved.

Reverse vaccinology tries to overcome problems related to the empirical identification of antigens. Previously, a combination of different bioinformatics prediction methods and *in vitro* testing has been used to identify T-cell epitopes in pigs

[5]. In addition, recent approaches that use immunoinformatic tools have been used to identify MHC class I and class II T-cell epitopes that are highly conserved in SwIVs circulating in the US swine population [6]. In the present study, reverse vaccinology technologies were extended to identify cross-reactive T-cell epitopes to two different H1N1 influenza A virus (IV) strains during the infection of pigs expressing SLA-1\*0702 allele. Strain A/swine/Spain/SF11131/2007 (SpH1N1) is an 'avian-like' H1N1 SwIV [7], whereas strain A/swine/Denmark/101310-1/2011 (pdmH1N1) is an H1N1 strain that circulated in pigs, but belongs to the 2009 pandemic lineage.

Firstly, T-cell epitopes residing in SpH1N1 and pdmH1N1 were predicted for SLA-1\*0702 binding using a previously described strategy that combines two methods for *in silico* prediction [8]. Briefly, the neural network NetMHCpan v. 2.8 prediction tool ([www.cbs.dtu.dk](http://www.cbs.dtu.dk)) and a positional scanning combinatorial peptide library (PSCPL) for the SLA-1\*0702 binding motif were used to predict T-cell epitopes. The experimental strategy of PSCPL has been described previously for both HLA [9] and SLA [10] proteins.

Received 13 October 2016; Accepted 16 February 2017

**Author affiliations:** <sup>1</sup>Centre de Recerca en Sanitat Animal (CRESA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, Bellaterra, Spain; <sup>2</sup>Technical University of Denmark, National Veterinary Institute (DTU VET), 1870 Frederiksberg C and 4771 Kalvehave, Denmark; <sup>3</sup>Statens Serum Institut, 5 Artillerivej, Copenhagen 2300, Denmark; <sup>4</sup>Centro de Investigación en Sanidad Animal, INIA, Valdeolmos 28130 Madrid, Spain; <sup>5</sup>The Pirbright Institute, Surrey GU24 0NF, UK.

**\*Correspondence:** Maria Montoya, [maria.montoya@pirbright.ac.uk](mailto:maria.montoya@pirbright.ac.uk)

**Keywords:** swine influenza virus; reverse vaccinology; MHC class I T cell epitopes; cross-reactive epitopes.

**Abbreviations:** d.p.b., days post boost; d.p.i., days post primary infection; IV, influenza virus; PSCPL, positional scanning combinatorial peptide library; SwIV, swine influenza virus.

†These authors contributed equally to this work.

Secondly, the peptide sequences that were consistently predicted by these two methods in combination were further analysed for actual MHC binding affinities ( $K_d$  less than 1000 nM) to recombinant SLA-1\*0702 (Table 1) by an *in vitro* immunosorbent assay [11]. Following these criteria, 11 sequences were predicted to be T-cell epitopes for the selected SLA allele and seven of them were conserved between both SwIV strains (Table 1). These sequences happened to be located within HA, NA and M1 proteins. Finally, tetramers were generated, as previously described [10, 12], using the 11 peptides identified *in vitro* and a specifically designed peptide as a negative control. Theoretically, the detection of T-cell populations by recognizing specific peptides presented on SLA-1\*0702 would indicate the recognition of viral T-cell epitopes.

Four pigs bearing the allele SLA-1\*0702 and a mismatched pig (not bearing the selected SLA allele) were selected following genomic SLA-I amplification by high-resolution sequence-specific primers [13] and infected with SpH1N1. All animals but one (L12) were intranasally infected with 3 ml, 9.04 logTCID<sub>50</sub> of SpH1N strain. L12, the SLA-I-matched pig, was intratracheally infected with the same dose. At 139 days post-primary infection (d.p.i.), animals were intranasally infected with 6 ml, 5.58 logTCID<sub>50</sub> of the Danish pdmH1N1 strain. The study was carried out in accordance with Danish legislation on animal experimentation and EU regulations on the use of laboratory animals for research (protocol number 2012-15-293–00682). All animals were euthanized 14 days post-second infection (boost; d.p.b.), corresponding to 153 d.p.i. Serum samples were collected to monitor antibody levels against both SwIVs by hemagglutination inhibition assay [14] and influenza A Ab ELISA (IDEXX). PBMCs were isolated from animals at different days post-infection. Biotinylated tetramers

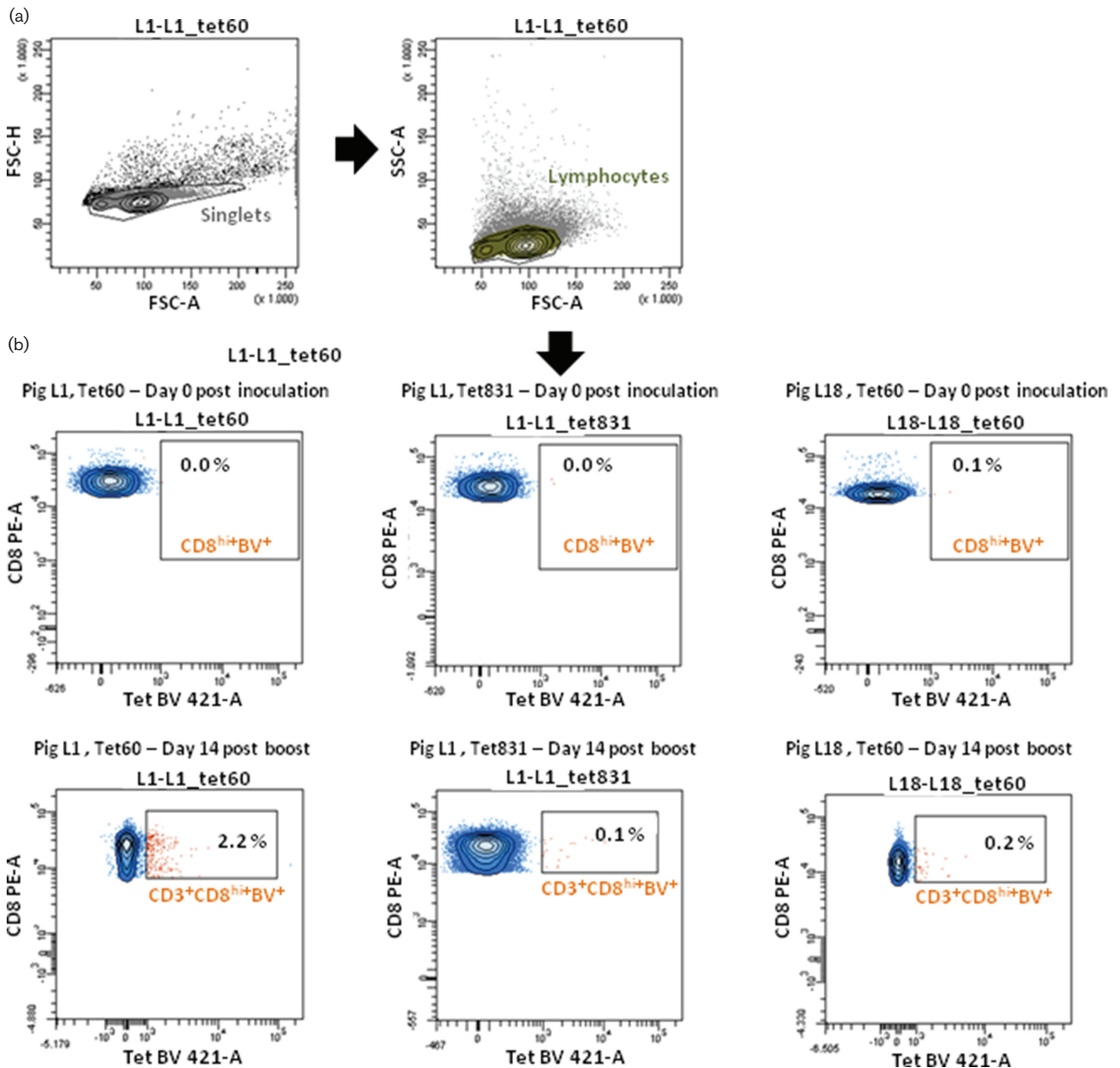
(Table 1) were used to stain the PBMCs and were visualized with a streptavidin-BV<sub>421</sub> fluorophore. PE-conjugated MAb against porcine CD8 $\alpha$  (clone 76-2-11, BD Pharmingen) and FITC-conjugated MAb against porcine CD3 $\epsilon$  (clone PPT3, Southern Biologend) were additionally used to characterize cells that stained positive with the tetramers. CD3<sup>+</sup>CD8<sup>high</sup> cells contain a CD8<sup>+</sup> $\alpha\beta$  T-cell subpopulation which recognises class-I epitopes [5, 15]. Tetramers showing frequencies two times higher compared to background (negative control or tetramer 831) and two times higher compared to the mismatched pig staining were regarded as positive.

None of the pigs developed clinical signs due to infection. Humoral SpH1N1- and pdmH1N1-specific responses were generated against each virus after infection (data not shown). Tetramer-positive T-cell subpopulations were below detection levels at 4, 7 and 9 d.p.b. (data not shown). At 14 d.p.b. specific T-cell responses were detected within the CD3<sup>+</sup>CD8<sup>high</sup> subpopulations (Tet<sup>+</sup>CD3<sup>+</sup>CD8<sup>high</sup>) as compared with the negative controls (Fig. 1, Table 2). The frequency of Tet<sup>+</sup>CD3<sup>+</sup>CD8<sup>high</sup> ranged from 0.4 to 2.6 %, depending on individual peptides and animals (Table 2). Nine peptides were identified as T-cell epitopes by tetramer staining; three located in the M1 protein (Tet62, Tet56 and Tet64), three in NA (Tet55, Tet60 and Tet59) and three in HA (Tet93, Tet57 and Tet66). These results highlighted the high efficiency of the discovery of class I restricted T-cell epitopes using this pipeline. The NA<sub>171–180</sub> (CPIGEVPSPY) peptide was considered to be immunodominant, as it was consistently detected in all pigs with the highest frequency within CD3<sup>+</sup>CD8<sup>high</sup> cells. Importantly, most of the other T-cell epitopes were only found in animal L6, in which NA<sub>171–180</sub> was not dominant.

**Table 1.** SwIV peptides *in silico* predicted to bind with SLA-1\*0702 with their respective *in vitro* binding results

Strong binding peptides with a  $K_d$  less than 1000 nM were used to generate tetramers.

Peptide sequence	Viral protein of origin (no.)	Position	Virus		Kd (nM)	Tetramer name
			pdmH <sub>1</sub> N <sub>1</sub>	SpH <sub>1</sub> N <sub>1</sub>		
NADTLCIGY	HA(1)	16–24	+		1 543	
SLSTASSWSY	HA(2)	86–95	+		35	57
TLYQNNHTY	HA(3)	207–215		+	6	66
YVSVGSSKY	HA(4)	215–223		+	987	93
SVKNGTYDY	HA(5)	295–303	+		20 000	
GMIDGWYGY	HA(6)	300–308		+	20 000	
EIGNGCFEFY	HA(7)	476–485	+	+	132	63
CPVSGWAIY	NA(1)	92–100	+	+	6	55
CPIGEVPSPY	NA(2)	171–180	+	+	37	60
GPSNGQASY	NA(3)	245–253	+	+	25	59
SVELNAPNY	NA(4)	266–274		+	20 000	
EMNAPNYHY	NA(5)	268–278	+		231	61
NMDRAVKLY	M1(1)	92–100	+	+	293	62
ALASCMGLIY	M1(2)	123–132	+	+	127	56
LASCMGLIY	M1(3)	124–132	+	+	9	64
VSAAAAAY	Negative control					831



**Fig. 1.** Tetramer staining of PBMCs of infected pigs. Example of gating strategy and staining of cells from pig L1 and pig L18 (mismatched pig) at days 0 and 153 post-infection (14 d.p.b.). (a) Singlet and lymphocyte analysis. (b) CD3<sup>+</sup>CD8<sup>high</sup> cells are shown. Tetramer 60 (Tet 60) is shown to specifically stain PBMCs from pig L1 at 14 d.p.b. The same tetramer and negative control tetramer (Tet 381) do not stain PBMCs at 0 d.p.i. or PBMCs from the mismatched pig (L18) at any time point.

Several factors can account for the different immunodominance hierarchy among animals [16]. For example, the pigs in this study were not inbred; therefore, different levels of SLA-1\*0702 might have been expressed on pig cells, depending on whether these animals were homozygous or heterozygous for this allele.

All T-cell epitopes identified in NA and M1 proteins were conserved between both IVs. By contrast, the T-cell epitopes

identified in HA were not conserved. However, it is noteworthy that SpH1N1-HA-specific T-cells (Tets 66 and 93) were detected after pdmH1N1 infection, indicating that a population of T-cells was primed after the second infection, which cross-reacted. A previous study in pigs suggested that there is flexibility in T-cell receptor recognition of class I epitopes and thus they can recognize non-homologous peptides [5]. It could also be speculated that HA-SpH1N1-specific T-cells, generated during the first infection, were

**Table 2.** Frequency of Tet<sup>+</sup>CD3<sup>+</sup>CD8<sup>+</sup> cells at 14 d.p.b. Numbers in bold show values two times higher than the background (negative control or tetramer 831)

Those values that were also two times higher than the respective peptide in the mismatched pig (L18) are underlined.

Pig	Frequency of tetramers											
	831	93	55	56	57	59	60	61	62	63	64	66
L1	0.1	0.1	0.1	<b>0.7</b>	<u>1.9</u>	0.2	<u>2.2</u>	0.0	0.0	0.1	<u>0.7</u>	0.0
L6	0.1	<u>0.4</u>	<u>1.7</u>	<u>1.2</u>	<b>1.2</b>	<u>2.1</u>	<u>1.0</u>	<b>0.4</b>	<u>0.7</u>	0.1	0.1	<u>1.0</u>
L11	0.7	0.1	0.2	0.0	1.2	1.2	<u>2.6</u>	0.6	1.3	0.1	0.8	0.7
L12	0.3	0.1	0.2	0.1	0.4	0.2	<u>0.7</u>	0.1	0.4	0.6	0.3	0.5
L18	0.1	0.1	0.4	0.5	0.8	0.7	0.2	0.2	0.1	0.1	0.1	0.3

re-activated by pdmH1N1 in a non-specific or bystander way, as has been described for other systems [17].

A recent study identified a number of SLA-I epitopes by using another immunoinformatic tool in pigs with a different SLA-I [6]. Interestingly, seven of the eleven selected epitopes in this study (HA1, HA2, HA5, HA6, NA5, M1-1 and M1-3) were identical to or had just one amino acid change compared to those reported by Gutiérrez *et al.* [6] using outbred pigs expressing SLA-I alleles other than SLA-1\*0702. The combination of these data suggests that these epitopes potentially have cross-reactive properties and may be relevant in a broad range of pigs. Out of these seven sequences, HA2 (Tet 57), NA5 (Tet 61), M1-1 (Tet 62) and M1-3 (Tet 64) were selected for the detection of epitope-specific T-cells (CD3<sup>+</sup>CD8<sup>+</sup>). All of them depicted specific T-cell subpopulations in some of the pigs, although they were not dominant. Remarkably, the majority of the predicted epitopes in this study and in Gutiérrez *et al.* [6] had tyrosine in position 9, suggesting that an amino acid with a hydrophobic side chain might be the preferable option for SLA-I antigen presentation in pigs.

In conclusion, the data in this work further extend the accuracy and versatility of our pipeline for SLA class I T-cell epitope identification and confirm the epitope predictions generated using other technologies. Several cross-reacting T-cell epitopes were identified and specific subpopulations were detected following heterologous SwIV infection. The functionality of these epitopes and their involvement in cross-protection will require further assessment in the future with the aim of applying these technologies to reverse vaccinology for SwIV in pigs.

#### Funding information

The research leading to these results received funding from the European Community's Seventh Framework Programme (FP7, 2007–2013), Research Infrastructures Action, under grant agreement no. FP7-228393 (NADIR project), and from the Spanish Government's MINECO AGL2013-22200-C02-01 and BBSRC BBS/E/1/00002014 grants.

#### Acknowledgements

We thank the staff in the animal facility for their support with the infection experiments. We also thank Dr Bryan Charleston at the Pirbright Institute for his support with this work and the manuscript. We thank Dr E. Reid for critically reviewing and editing the manuscript.

#### Conflicts of interest

The authors declare that there are no conflicts of interest.

#### References

1. Watson SJ, Langat P, Reid SM, Lam TT, Cotten M *et al.* Molecular epidemiology and evolution of influenza viruses circulating within European swine between 2009 and 2013. *J Virol* 2015;89: 9920–9931.
2. Crisci E, Mussá T, Fraile L, Montoya M. Review: influenza virus in pigs. *Mol Immunol* 2013;55:200–211.
3. Busquets N, Segalés J, Córdoba L, Mussá T, Crisci E *et al.* Experimental infection with H1N1 European swine influenza virus protects pigs from an infection with the 2009 pandemic H1N1 human influenza virus. *Vet Res* 2010;41:74.
4. Qiu Y, de Hert K, van Reeth K. Cross-protection against European swine influenza viruses in the context of infection immunity against the 2009 pandemic H1N1 virus: studies in the pig model of influenza. *Vet Res* 2015;46:105.
5. Pedersen LE, Breum SØ, Riber U, Larsen LE, Jungersen G. Identification of swine influenza virus epitopes and analysis of multiple specificities expressed by cytotoxic T cell subsets. *Virology* 2014;11:163.
6. Gutiérrez AH, Loving C, Moise L, Terry FE, Brockmeier SL *et al.* *In vivo* validation of predicted and conserved T cell epitopes in a swine influenza model. *PLoS One* 2016;11:e0159237.
7. Baratelli M, Córdoba L, Pérez LJ, Maldonado J, Fraile L *et al.* Genetic characterization of influenza A viruses circulating in pigs and isolated in North-East Spain during the period 2006–2007. *Res Vet Sci* 2014;96:380–388.
8. Pedersen LE, Rasmussen M, Harndahl M, Nielsen M, Buus S *et al.* A combined prediction strategy increases identification of peptides bound with high affinity and stability to porcine MHC class I molecules SLA-1\*04:01, SLA-2\*04:01, and SLA-3\*04:01. *Immunogenetics* 2016;68:157–165.
9. Stryhn A, Pedersen LO, Romme T, Holm CB, Holm A *et al.* Peptide binding specificity of major histocompatibility complex class I resolved into an array of apparently independent subspecificities: quantitation by peptide libraries and improved prediction of binding. *Eur J Immunol* 1996;26:1911–1918.
10. Pedersen LE, Harndahl M, Rasmussen M, Lamberth K, Golde WT *et al.* Porcine major histocompatibility complex (MHC) class I molecules and analysis of their peptide-binding specificities. *Immunogenetics* 2011;63:821–834.
11. Sylvester-Hvid C, Kristensen N, Blicher T, Ferré H, Lauemøller SL *et al.* Establishment of a quantitative ELISA capable of determining peptide–MHC class I interaction. *Tissue Antigens* 2002;59: 251–258.
12. Leisner C, Loeth N, Lamberth K, Justesen S, Sylvester-Hvid C *et al.* One-pot, mix-and-read peptide-MHC tetramers. *PLoS One* 2008;3:e1678.

13. Pedersen LE, Jungersen G, Sorensen MR, Ho CS, Vadekær DF. Swine Leukocyte Antigen (SLA) class I allele typing of Danish swine herds and identification of commonly occurring haplotypes using sequence specific low and high resolution primers. *Vet Immunol Immunopathol* 2014;162:108–116.
14. Van Reeth K, Brown IH, Dürrwald R, Foni E, Labarque G et al. Seroprevalence of H1N1, H3N2 and H1N2 influenza viruses in pigs in seven European countries in 2002–2003. *Influenza Other Respir Viruses* 2008;2:99–105.
15. Gerner W, Talker SC, Koinig HC, Sedlak C, Mair KH et al. Phenotypic and functional differentiation of porcine  $\alpha\beta$  T cells: current knowledge and available tools. *Mol Immunol* 2015;66:3–13.
16. Yewdell JW. Confronting complexity: real-world immunodominance in antiviral CD8<sup>+</sup> T cell responses. *Immunity* 2006;25:533–543.
17. Chu T, Tyznik AJ, Roepke S, Berkley AM, Woodward-Davis A et al. Bystander-activated memory CD8 T cells control early pathogen load in an innate-like, NKG2D-dependent manner. *Cell Rep* 2013;3:701–708.

**Five reasons to publish your next article with a Microbiology Society journal**

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as 'excellent' or 'very good'.
5. Your article will be published on an interactive journal platform with advanced metrics.

**Find out more and submit your article at [microbiologyresearch.org](http://microbiologyresearch.org).**