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Expansion of Proteome-wide *Coxiella burnetii* Comparative T-cell Epitope Prediction to Include Small Ruminant Hosts

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Abstract

Background: *Coxiella burnetii* is the causative agent of Q fever, a human disease that can be acquired from livestock. Diseases caused by this organism have caused great losses in livestock and human health. No vaccine is approved for use in the United States, and formalin-inactivated whole-cell vaccines pose a significant manufacturing risk for biocontainment. A subunit vaccine using recombinant peptides from *C. burnetii* would be safer and less resource-intensive to produce. This study used reverse vaccinology to expand our prediction sets of T-cell epitopes for the major histocompatibility complex (MHC) Class I and II alleles of cattle, sheep, and goats. Thereafter, the present results were compared with those from our previous prediction sets for mice and humans.

Results: Small ruminant breed representation for the United States was ensured by querying whole genome sequences on the National Center for Biotechnology Information database. Consequently, twenty-two sheep MHC Class I, seventeen goat MHC Class I, and one goat MHC Class II alleles were added to the analyses, resulting in a total of fifty-six sheep MHC Class I, eighteen goat MHC Class II, and twenty-seven goat MHC Class II alleles. Predicted interactions of *C. burnetii* proteome-derived peptides with each MHC allele were categorized as strong, weak, or non-binding based on bioinformatic scores. Interspecies comparisons resulted in 256 peptides of interest for MHC Class II presentation and 766 peptides of interest for MHC Class I presentation. Of these, 51 peptides were predicted to bind with both classes of MHC alleles, of which 33 were newly identified.

Conclusion: The high scoring T-cell epitope predictions identified in this study provide grounds for prioritizing subunit candidates to further develop a safe and perhaps broadly effective *C. burnetii* vaccine.

Keywords: *Coxiella burnetii*, T-cell epitope, Small ruminant, Bioinformatics, Reverse vaccinology

Introduction

Subunit vaccines contain specific peptides known to confer protection, alleviate disease symptoms, or prevent spread [1-4]. This approach to vaccine development requires knowledge about which pathogen peptides elicit such responses in the vaccinated host. Without this foundational knowledge, vaccine development proceeds fastest when the pathogen of interest has a small proteome or an antibody-mediated protective response. An example of this is influenza, where antibodies against hemagglutinin or neuraminidase reduce disease symptoms, spreading, and time course [4,5]. On the other hand, effective immune responses to some pathogens require cell-mediated immunity, and direct peptide

investigation for many pathogens is hampered by the sheer size of their proteomes [6-10]. One such pathogen is *Coxiella burnetii*, a bacterium that produces thousands of proteins and for which effective defenses against infection in humans and animals requires cell-mediated immune responses [11, 12].

In humans, exposure to *C. burnetii* can result in several outcomes, including asymptomatic transient infection, acute illness, or persistent infection [11,13]. Estimation of case outcomes would suggest that 40% of exposed humans respond with acute flu-like illness while the rest of the exposed population are asymptomatic [11,13,14]. Chronic Q fever and persistent Q fever are terms used to designate long-term symptomatic infections in humans [11,13], which commonly present as either endocarditis, osteoarticular infection, hepatitis, or vascular infection [11,13,14]. Infections in animals may also remain asymptomatic but the most common clinical outcome is reproductive disease [12,15-17]. Importantly, asymptomatic infections in domestic ruminants can still result in high bacterial shedding in their birth products [15,16,18]. Thus, *C. burnetii* is of concern to both animal and human health, and the risk of exposure is most critical in the vicinity of periparturient domestic ruminants.

Coxiella burnetii is a Gram-negative bacterium that readily persists within the environment in a spore-like form and is considered endemic in many regions of the world [19,20]. The respiratory tract is the most common route of exposure for animals and humans alike, and by which the minimum infective dose is very low [18,21]. In light of these facts and the risk of severe clinical outcomes, *C. burnetii* is classified as a Biosafety Level 3 (BSL3) organism [21]. Thus, producing a formalin-inactivated whole-cell *C. burnetii* vaccine requires expensive BSL3 laboratories and procedures and has not been approved in the United States. Without an effective vaccine, endemic areas like the United States remain at high risk for outbreaks [22-24]. An epidemic outbreak of *Coxiella burnetii* occurred in the Netherlands between 2007 and 2010, which cost the country millions of dollars to treat human cases and implement emergency animal intervention strategies [25]. It is therefore prudent to continue efforts toward developing an effective *C. burnetii* vaccine that can be efficiently and safely produced.

Reverse vaccinology is a technique that has gained interest in recent years [4,9,26]. This technique relies on bioinformatic tools to assess the ability of pathogen proteins to activate the host's immune system. Activation of T-cells relies on only the primary structure (i.e., linear sequence) of a pathogen protein, whereas B-cell activation can require recognition of higher-order tertiary protein structure. Further, T-cell activation relies on the interaction between a given pathogen peptide and the major histocompatibility complex (MHC), a complex of host proteins that establish an adaptive immune response by presenting peptides of pathogen proteins to T-cells. Peptides that interact with MHCs are designated T-cell epitopes. There are two basic types of T-cells, wherein each responds to a different allelic class of MHC molecule. MHC Class I molecules present bound peptides to 'cytotoxic' ($CD8^+$) T-cells whereas MHC Class II molecules present bound peptides to 'helper' ($CD4^+$) T-cells. By utilizing MHC eluted ligands and known amino acid interaction preferences between peptides and MHC alleles, modern bioinformatic tools mine the exceptionally large sequence space of pathogens with large proteomes for peptides that are most likely to activate T-cell mediated host immune responses.

Bioinformatic tools have been recently expanded to include MHC alleles of cattle [27,28]. Our group previously applied these tools to predict *C. burnetii* peptides that bind the known sequences of MHC Class I in cattle, as well as MHC Class I and Class II alleles within the mouse and human species [29]. A bovine MHC Class II prediction tool, NetBoLAIIpan, has subsequently become available [27]. In the present analysis, NetBoLAIIpan is employed to expand predictions for cattle, sheep, and goat T-cell epitopes within the *C. burnetii* proteome. In addition, NetMHCpan was utilized to predict MHC Class I T-cell epitopes for sheep and goat alleles.

Methods

Random peptide generation

Protein sequences were randomly generated using the Expasy RandSeq tool [30], where sequences were produced using the average amino acid composition present on Swiss-Prot. Generated proteins were separated into 13-mer, 14-mer, 15-mer, 16-mer, or 17-mer peptides to be scrambled. Mimotopes Scrambled tool was exploited to scramble supplied amino acids one thousand times [31]. Forty-thousand peptides were produced for each peptide length, making a total of 200,000 randomly generated peptides to train NetBoLAIIpan 1.0 for small ruminant allele evaluation (Personal Communication).

MHC Class I epitopes are usually shorter than MHC Class II epitopes [27,32-34], so downsizing of random epitopes generated for MHC Class II was used to supply MHC Class I epitopes. Generation of random peptides consisting of 8-mers, 9-mers, 10-mers, and 11-mers was accomplished using the longer random peptide sequences. Specifically, peptides from 13-mer, 14-mer, 15-mer, and 16-mer files had 5 amino acids removed from the C-terminal end to generate 160,000 random peptides used to train NetMHCpan 4.1.

Identification of MHC Class I and MHC Class II alleles from sheep and goat whole genome sequences

NCBI was assessed for whole genome sequences pertaining to domestic sheep and goat species. Present whole genome sequences (WGS) were only included in the analysis if the assembly had reached chromosome level, 25X coverage,

and was in a breed of interest (**Supplemental Table 1**). **Supplemental Table 1** contains the species, breed, chromosome accession number, and MHC classes evaluated within present WGSs. Isolated WGSs were evaluated for MHC alleles within chromosome 20 for domestic sheep species and within chromosome 23 for domestic goat species, as these chromosomes are known to house the MHC haplotypes [35]. Tblastn was employed to assess ten million base pairs of genome sequence at a time for the presence of MHC alleles. The protein query used was Ovar-N*01:01 (CAI43967.2) for MHC Class I within domestic sheep, ABQ14768.1 for MHC Class I within domestic goat, and Cahi-DRB1*01:01 (SPC50560.1) for MHC Class II within domestic goat [36-39]. Outputs were scrutinized for genomic regions which might house MHC alleles using the graphics feature, where an overall hit that had an identity above 50% was scrutinized for a grouping of hits within the graphics feature. If one of the hits within a group had an identity above 78%, then the million base pair region was marked for further analysis.

Following notation of these regions, Genscan was used to translate DNA [40]. Predicted proteins were queried using the Basic Local Alignment Search Tool for protein (BLASTp) and the previously mentioned MHC Class I or MHC Class II alleles. Returned hits were scrutinized for percent identity and percent coverage indicative of true MHC alleles. To determine cut-off values, known Immuno Polymorphism database (IPD)-MHC database alleles were queried using BLASTp. This analysis determined that true sheep MHC Class I alleles tend to have a percent identity above 78% and a percent coverage above 80%. Concurrently, goat MHC Class II alleles were found to maintain a percent identity above 75% and a percent coverage of 30%. However, when assessing translated proteins comparatively against Cahi-DRB1*01:01, a percent coverage of 80% was employed due to most of the annotated goat MHC Class II alleles being partially sequenced. With only one prior MHC Class I allele present for goat species, a percent identity of 75% and a percent coverage of 80% was employed to follow what was determined for goat MHC Class II alleles.

Following MHC allele identification, pairwise distance analysis of allele proteins from the IPD-MHC database, prior publications, and WGS inquiry was conducted [36-38,40-50]. Pairwise distance used MEGA X software version 10.1.8, where exons 2 and 3 were aligned for MHC Class I alleles and exon 2 was aligned for MHC Class II alleles [51]. Resultant pairwise distance charts are present in **Supplemental Files 1** (MHC Class II) and **2** (MHC Class I). A pairwise distance of 0.0 indicated that tested alleles maintained 100% identity. Alleles that were not previously described but identified during whole genome scrutiny have their genomic locus listed as LP within **Supplemental Tables 2-4**; as well as in **Supplemental File 3**.

Selection of sheep MHC Class II alleles

Due to the plethora of MHC Class II alleles (130 Ovar-DRB1) present on IPD-MHC and having to train each allele within the NetBoLAIIpan 1.0 program, only a subset was chosen for analysis. Sheep MHC Class II alleles were selected based off breed association, frequency within populations, and phylogenetic distance present within published articles [42-44,46-50,52]. A list of the collected alleles and associated breeds of interest are present in **Supplemental Tables 2** and **3**.

NetBoLAIIpan version 1.0

Recent release of NetBoLAIIpan 1.0 allows for cattle MHC Class II alleles to be tested for interaction with 15-mer peptides [27]. Present on this database and program are 299 cattle MHC Class II alleles (**Supplemental Table 2**), which align with the DRB3 locus within the cattle genome. Run proteins were previously described in Piel et. al [29] and were used here to assess cattle MHC Class II allele interaction with the conserved *Coxiella burnetii* proteome.

To more accurately assess small ruminant MHC Class II alleles for interaction with *C. burnetii* peptides, the NetBoLAIIpan 1.0 program needed to be trained for each allele of interest by running the 200,000 randomly generated peptides per allele. The DRA gene is typically invariant or nearly so [53,54]. Importantly, the DRA allele used within NetBoLAIIpan 1.0 cannot be altered; therefore, each of the small ruminant MHCII alleles was run using the cattle MHC Class II-DRA and a small ruminant MHC Class II-DRB. Differences between ruminant MHC Class II DRA alleles were defined by protein alignment using ClustalW within the MEGA X software, employed gap and extension penalties were not altered from program defaults. Percent identity was calculated by using the base parameters on BLASTp. Following training, the small ruminant MHC Class II DRB alleles listed in **Supplemental Table 3** were tested for T-cell epitopes using the conserved *C. burnetii* proteome.

Following acquisition of NetBoLAIIpan 1.0 output files for all ruminant species, each file was loaded into the Sequel Server Management Studio (SSMS) v18.6. For cattle, tested MHC Class II alleles had percent rank values calculated by NetBoLAIIpan 1.0. Percent rank values were used to determine if a tested peptide bound strongly ($x < 2$) or weakly ($2 \leq x < 10$) to a given allele. This follows the scoring previously used [29] to define *C. burnetii* T-cell epitopes within the human and mouse species. The percent rank for small ruminant species was not calculated by NetBoLAIIpan 1.0; instead, the random peptide scores were employed per allele to determine the score associated with the top 2% and top 10% ranked hits. Thereafter, these score denominators were used to define weak and strong binders as done for the cattle species.

NetMHCpan version 4.1

Prior analysis has occurred for the 105 Bovine Leukocyte Antigen (BoLA) MHC Class I alleles present on NetMHCpan 4.1

[29]. Expansion of this dataset to include small ruminants MHC Class I alleles required running 160,000 randomly generated peptides for each allele of interest. Thereafter, each MHC Class I allele defined for sheep and goat species was analyzed against the conserved *C. burnetii* proteome. Similar to NetBoLAIIpan analysis, the output data files resulting from random peptide analysis were loaded onto a SSMS database. However, for MHC Class I analysis, the scores associated with the top 0.5% and 2% random peptides were defined, where a strong interaction had a percent rank < 0.5 and a weak interaction had a percent rank ≥ 0.5 and < 2.

High scoring peptides from NetBoLAIIpan 1.0 and NetMHCpan 4.1

Within the SSMS databases, percent ranks rendered each peptide:allele interaction as strong, weak, or non-existent. Thereafter, each peptide tested had the number of interactions between every allele summed. Within these summations, peptides which interacted with 45% of the tested alleles strongly or interacted with 90% of the alleles tested were isolated as high scoring peptides for MHC Class II alleles. Alternatively, MHC Class I high scoring peptides were identified as those which interacted with 60% of the alleles tested or were suggested to strongly interact with 45% of the alleles tested. Once the lists of high scoring peptides were calculated, they were scrutinized for peptides which overlapped by 50%. If given peptides overlapped, then the overlapping peptide(s) were removed. Removal of overlapping peptides prioritized cattle strong binding scores due to the bioinformatic program being trained on cattle elution data [27]. If the strong binding scores were the same, then the total number of alleles bound was scrutinized. This resolved most overlapping situations, but the few that required further discrepancy relied on peptide:allele scores from the ovine species.

Results

T-cell epitopes predicted for MHC Class II alleles of cattle

NetBoLAIIpan predicts pathogen peptides that interact with known cattle MHC Class II alleles, designating interacting peptides as potential T-cell epitopes [27]. There are multiple MHC Class II genes within chromosome 23 of cattle, but the DRB3 locus represents the most polymorphic and best studied [45,55-66]. Therefore, the NetBoLAIIpan program was initiated with DR alleles but not DQ or DY alleles. At the time of study, there were 299 Bovine Leukocyte Antigen (BoLA)-DRB3 alleles loaded onto the NetBoLAIIpan website. Pairwise differentiation of the BoLA-DRB3 alleles removed 4 that were identical in amino acid sequence (**Supplemental File 1**).

The remaining 295 MHC Class II alleles were tested for interaction with the previously defined conserved *C. burnetii* proteome consisting of 1,022 proteins [29]. These proteins were broken into 15-mer peptides, resulting in 293,520 peptides that were tested. Of these peptides, 55.8% were predicted to interact with at least one MHC Class II allele. However, only 6,155 peptides were considered of interest because of strong binding predicted with 45% of the alleles (133) or because of either strong or weak binding predicted with 90% of the alleles (266). One-hundred fifty peptides were predicted to bind either strongly or weakly with all the alleles tested (**Supplemental Table 5**). Three peptides were predicted to bind strongly with 99% of the cattle alleles (**Table 1**); however, the start position of the 15-mer peptides of the protein PanC differ by only one amino acid, 240 and 241, and so, are likely comprised of the same T-cell epitope.

Comparison of ruminant DRA proteins

The MHC Class II molecule is composed of two glycoproteins, the alpha and beta chains [4,26,35], which together form a peptide binding groove [1,26]. The alpha chains (DRA proteins) tend to be monomorphic whereas the beta chains (DRB proteins) are highly polymorphic [53-56,59,60,62,65]. Designed as a bioinformatic tool for cattle, the NetBoLAIIpan program holds the DRA protein constant but allows the user to choose the DRB protein against which peptides can be assessed for binding. However, the phylogenetic relationship between cattle and small ruminants is close and is reflected in the high sequence identity of cattle, sheep, and goat DRAs (**Figure 1**). As calculated using the BLASTp, the sheep and goat DRA proteins respectively share 96.8% and 96.1% identity with the cattle DRA protein. When comparing only the alpha 1 domain of DRA, that is the portion of DRA that participates

Table 1. Peptides predicted to strongly bind 292 MHC Class II alleles found in cattle.

Position	Peptide	GenbankID	SB	WB	TB	Gene Name	Locus Tag	Location
240	V**GDIRLIDNIPFAKD**	AAO89975.1	292	3	295	*panC*	CBU_0423	CYTOPLASM
241	**GDIRLIDNIPFAKD**K	AAO89975.1	292	3	295	*panC*	CBU_0423	CYTOPLASM
252	DSYLKYAPIHAVGAP	AAO91502.1	292	3	295		CBU_2013	CYTOPLASM

Position indicates the start of the peptide within the protein of interest, where the first amino acid in a protein is labeled position 1. Genbank ID, gene name, and locus tag represent protein information obtained from NCBI in the Nine Mile RSA 493 assembly. Protein location was previously predicted using the Inmembrane program [67]. The overlapping amino acids of the PanC peptides are indicated in bold and underlined. Abbreviations: SB: Strong Binders; WB: Weak Binders; TB: Total Binders.

DRA Cattle Allele (DAA16455.1)	M	A	I	T	R	V	P	I	L	G	L	F	I	T	V	L	I	G	L	Q	E	S	W	A	**I**	**K**	**E**	**N**	**H**	**V**
DRA Goat Allele (BAA23385.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	L	.	.	.	.	.	D	.	.
DRA Sheep Allele (CAX17684.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.	.	.	D	.	.
DRA Cattle Allele (DAA16455.1)	**I**	**I**	**Q**	**A**	**E**	**F**	**Y**	**L**	**K**	**P**	**E**	**E**	**S**	**A**	**E**	**F**	**M**	**F**	**D**	**F**	**D**	**G**	**D**	**E**	**I**	**F**	**H**	**V**	**D**	**M**
DRA Goat Allele (BAA23385.1)	.	.	.	.	.	.	.	.	N	.	.	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Sheep Allele (CAX17684.1)	.	.	.	.	.	.	.	.	N	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Cattle Allele (DAA16455.1)	**G**	**K**	**K**	**E**	**T**	**V**	**W**	**R**	**L**	**P**	**E**	**F**	**G**	**H**	**F**	**A**	**S**	**F**	**E**	**A**	**Q**	**G**	**A**	**L**	**A**	**N**	**M**	**A**	**V**	**M**
DRA Goat Allele (BAA23385.1)	Q	.	.	.	.	.	.	.	.	.	.	.	.	R	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Sheep Allele (CAX17684.1)	Q	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Cattle Allele (DAA16455.1)	**K**	**A**	**N**	**L**	**D**	**I**	**M**	**I**	**K**	**R**	**S**	**N**	**N**	**T**	**P**	**N**	**T**	**N**	V	P	P	E	V	T	L	L	P	N	K	P
DRA Goat Allele (BAA23385.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Sheep Allele (CAX17684.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Cattle Allele (DAA16455.1)	V	E	L	G	E	P	N	T	L	I	C	F	I	D	K	F	S	P	P	V	I	S	V	T	W	L	R	N	G	K
DRA Goat Allele (BAA23385.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
DRA Sheep Allele (CAX17684.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	I
DRA Cattle Allele (DAA16455.1)	P	V	T	D	G	V	S	Q	T	V	F	L	P	R	N	D	H	L	F	R	K	F	H	Y	L	P	F	L	P	T
DRA Goat Allele (BAA23385.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Sheep Allele (CAX17684.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	D	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Cattle Allele (DAA16455.1)	T	E	D	V	Y	D	C	K	V	E	H	L	G	L	N	E	P	L	L	K	H	W	E	Y	E	A	P	A	P	L
DRA Goat Allele (BAA23385.1)	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Sheep Allele (CAX17684.1)	.	.	.	.	.	.	.	.	.	.	.	W	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Cattle Allele (DAA16455.1)	P	E	T	T	E	N	A	V	C	A	L	G	L	I	V	A	L	V	G	I	I	A	G	T	I	F	I	I	K	G
DRA Goat Allele (BAA23385.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Sheep Allele (CAX17684.1)	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
DRA Cattle Allele (DAA16455.1)	V	R	K	A	N	T	V	E	R	R	G	P	L																	
DRA Goat Allele (BAA23385.1)	.	.	.	.	.	.	.	.	.	.	.	.	.																	
DRA Sheep Allele (CAX17684.1)	.	.	.	.	.	.	.	.	G	.	.	.	.																	

Figure 1. DRA protein alignment within ruminant species of interest. Alignment of the DRA protein from cattle, goats, and sheep [38,52,56]. The identifier in parenthesis is the Genbank ID for the aligned protein. Each cell contains an amino acid or a decimal which indicates no change from the cattle DRA reference protein. The bolded and underlined amino acids within the reference protein mark the alpha 1 domain of the protein.

in forming the peptide groove [26,56,66], the sheep and goat sequences respectively share 96.4% and 94.1% identity with cattle DRA (underlined and bolded in **Figure 1**). As a reference, the human DRA protein (GenbankID P01903.2) shares 80.71% identity with cattle DRA. Thus, despite the software limitation, we anticipate the pairing of small ruminant DRB proteins with the cattle DRA protein predicts T-cell epitopes that are robust to these few DRA differences between these species.

T-cell epitopes predicted for MHC Class II alleles of sheep and goat

The sheep DRB alleles located on the Immuno Polymorphism Database (IPD)-MHC database have been identified within breeds prominent in the United States, including the Suffolk, Rambouillet, Polypay, Columbia, and Texel (**Supplemental Table 2**) [68]. In contrast, many of the MHC Class II DRB goat alleles are from the Scottish Cashmere goat, an infrequent breed within the United States [37]. Therefore, the whole genome sequences present on the National Center for Biotechnology Information (NCBI) database for Saanen and San Clemente goat breeds were examined for MHC Class II DRB alleles within chromosome 23. As shown in **Supplemental Table 3** and **Supplemental File 1**, two alleles aligned with previously defined proteins and one *de novo* allele was established.

After training the software on 200,000 randomly generated peptides, the strength of interactions of each MHC Class II allele from sheep (21 DRB alleles) and goat (27 DRB alleles) with the conserved *C. burnetii* proteome [29] were predicted. There were 10,932 peptides predicted to interact with sheep alleles and 10,058 peptides predicted to interact with goat alleles. There were 5,618 high scoring peptides predicted to interact with MHC Class II molecules of sheep, goat, and cattle (**Figure 2**). This list reduced to 2,389 predicted MHC Class II T-cell epitopes for all three species when peptides overlapping by 7 amino acids were removed; this list comprises 0.8% of all peptides examined (**Supplemental Table 6**).

Pan-allelic peptides represent those predicted to bind all alleles tested. Pan-allelic strong binding was predicted for 287 peptides in sheep and 54 peptides in goats, 49 of which were pan-allelic strong binders in both sheep and goats. In including peptides predicted to strongly bind 95% of the cattle alleles (280 out of 295), eleven peptides were predicted to bind strongly with a great majority of the MHC Class II alleles tested across these three ruminant species (**Table 2**). Notably, each of these peptides are part of proteins likely to be localized to the bacterial cytoplasm.

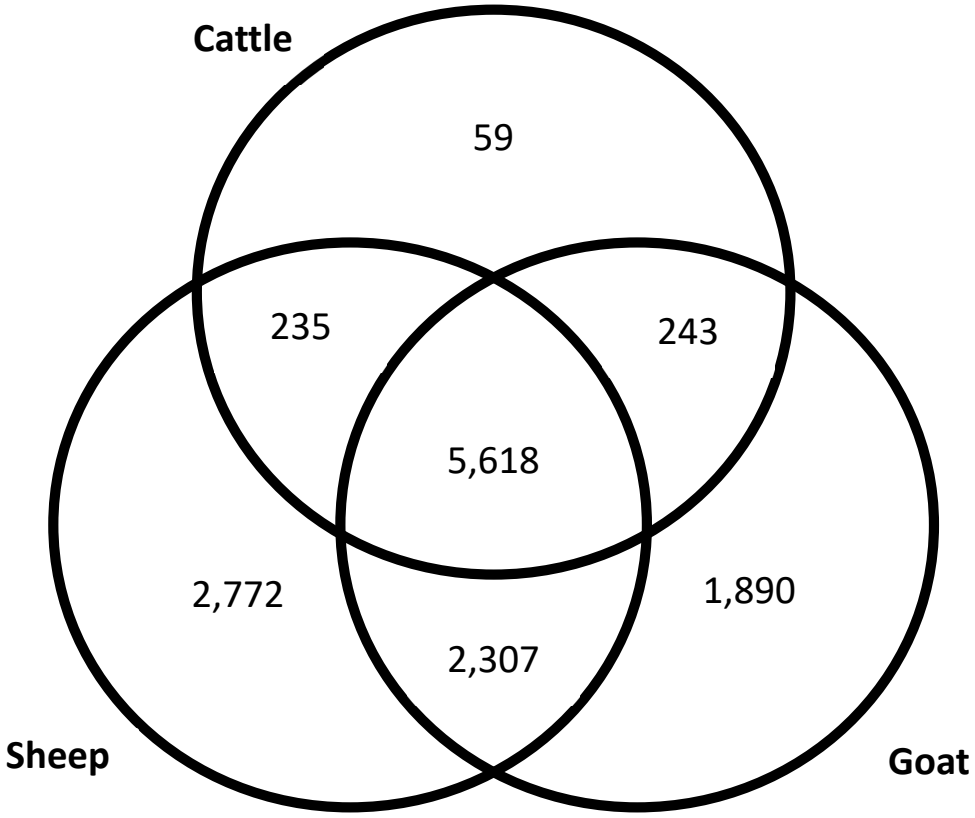


Figure 2. Predicted peptide interaction numbers for ruminant MHC Class II molecules. Venn Diagram depicting the number of predicted MHC Class II T-cell epitopes within each ruminant species and if peptides were predicted to interact with multiple species.

Table 2. T-cell epitopes predicted to strongly bind most MHC Class II molecules currently reported in ruminants*.

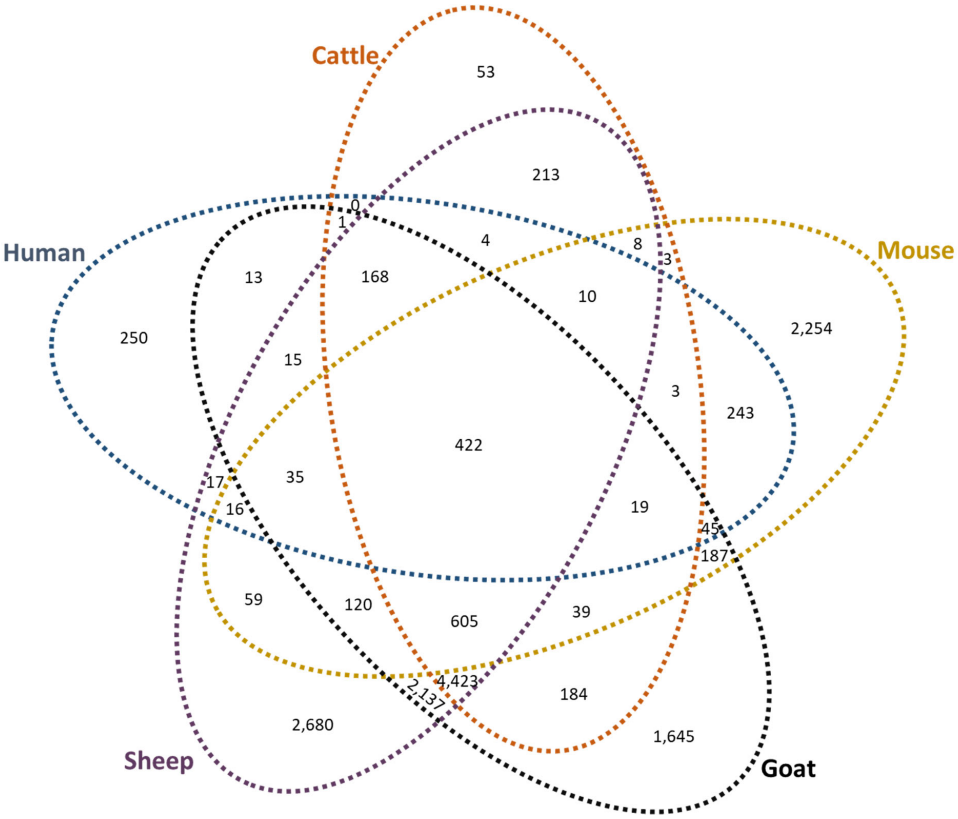
Position	Peptide	GenbankID	Gene Name	Locus Tag	Location
103	AAKKIIKLIKDQKLK	AAO89678.1	*yajQ*	CBU_0114	CYTOPLASM
105	IKRVKIFAAGKLEKP	AAO89815.1	*rplO*	CBU_0257	CYTOPLASM
240	VGDIRLIDNIPFAKD	AAO89975.1	*panC*	CBU_0423	CYTOPLASM
37	PVGFEIAQALHLPLD	AAO90019.1		CBU_0470	CYTOPLASM
31	DKSLVAIKNVTVNEP	AAO90158.1	*fabZ*	CBU_0614	CYTOPLASM
275	ISSFVPIEIHVIPER	AAO90229.1		CBU_0685	CYTOPLASM
104	GEPFKYVVNFEVYPE	AAO90277.1	*tig*	CBU_0737	CYTOPLASM
56	LSTIFIAVPIHHFKN	AAO90505.1		CBU_0984	CYTOPLASM
581	DIPFHAVEIEKLAHR	AAO90739.1		CBU_1230	CYTOPLASM
252	DSYLKYAPIHAVGAP	AAO91502.1		CBU_2013	CYTOPLASM
108	YDSIIKFAKANKLRI	ACI15237.1		CBU_0095a	CYTOPLASM

* Strong binding predicted for 292 of 295 known DR cattle alleles and all tested alleles for sheep and goats.
GenbankID, gene name, and locus tag annotations come from NCBI, where location was predicted by Inmembrane. The peptide and position are generated by NetBoLaIIpan.

Comparison of MHC Class II T-cell epitopes between ruminant, human, and mouse species

All data from the present and prior [29] studies are maintained on the same SQL Server Management Studio (SSMS) data server, which allowed us to readily compare T-cell epitope predictions across species. Peptides with exceptional binding characteristics for mouse and human MHC alleles were previously identified [29]. Similarly, for cattle, sheep, and goat MHC alleles, peptides were identified as having exceptional binding characteristics if predicted to strongly bind 45% or at least weakly bind 90% of the alleles tested within one of these species. This returned 15,871 peptides with exceptional within-species binding characteristics, the between species intersections of which are shown in **Figure 3A**. It is interesting to note that the largest number of peptides bound either only sheep, only goat, only mouse, sheep and goat, or sheep and goat and cattle alleles. It must be recognized that the criterion for making such distinctions is subject to the predominance of alleles in the populations of interest. This most likely affects the distinctions as applied to the relatively few alleles yet known for small ruminants and

A.



B.

total peptides of interest	6,155	10,058	10,932	4,068	1,261
% same as cattle	cattle	58.3%	53.5%	27.3%	49.7%
% same as goat	95.2%	goat	72.5%	36.2%	56.9%
% same as sheep	95.1%	78.8%	sheep	31.3%	54.5%
% same as mouse	18.0%	14.6%	11.7%	mouse	62.9%
% same as human	10.2%	7.1%	6.3%	19.5%	human

Figure 3. Predicted peptide interaction numbers for MHC Class II molecules from all species. (A) Peptide numbers which reached high scoring cut-offs within each species (human, mouse, cattle, goat, and sheep) of interest for MHC Class II allelic interaction. The Venn Diagram breaks out the predicted peptides by how they interacted between species. **(B)** Percent of peptides predicted to interact between species in a direct species to species comparison. The top row indicates the total number of high scoring predicted MHC Class II peptide interactions per species. Percentage is color coded for ease, where blue is a high number of predicted peptides between species and red is the opposite.

the eight alleles encompassed by NetMHCIIpan for mice. The increased peptide numbers predicted to bind all ruminant species supports their close evolutionary relationship. **Figure 3B** depicts the percent of peptides which remain consistent during direct species to species comparison, where ruminants share predicted interactions of greater than 50% of peptides in all cases. Nonetheless, there were 422 peptides identified with exceptional MHC allelic coverage for all five species, 256 of which differed by at least 50% in linear sequence identity (**Supplemental Table 7**).

Analysis of the pan-allelic T-cell epitopes predicted to interact with sheep, goat, and human species was decided upon due to human health being less affected by cattle exposure, following introduction of milk pasteurization [69], and since mice are less important to vaccine development for sheep, goats, and humans. Peptides which bound all goat or sheep alleles strongly or which are predicted to interact with all human alleles were filtered out of the data set (**Table 3**). As can be seen, 4 of the 11 T-cell epitopes described in **Table 2** are present in the resultant dataset from the small ruminant species. In contrast to this, when assessing peptides which interacted with all human alleles tested, there were none that overlapped with high scoring ruminant peptides (**Table 2** versus **Table 3**). However, two peptides remained consistent when assessing the dataset for human and small ruminant T-cell epitopes that interacted with all alleles tested. Filtering the data with these criteria returned proteins that localized beyond the cytoplasm within the membrane or that are secreted.

Table 3. Pan-allelic T-cell epitopes predicted for small ruminant or human MHC Class II alleles

Position	Peptide	GenbankID	Species	SB	WB	TB	Gene Name	Locus Tag	Location
Peptides binding all small ruminant MHC Class II alleles									
105	KKIIKLIKDQKLKVQ	AAO89678.1	Cow	288	7	295	*yajQ*	CBU_0114	CYTOPLASM
			Human	102	62	164			
			Mouse	5	0	5			
152	KKNFYQLPINKVIDR	AAO89725.1	Cow	246	41	287		CBU_0165	Membrane
			Human	98	47	145			
			Mouse	4	4	8			
197	QMEFTAIRINPVVAG	AAO90399.1	Cow	271	22	293		CBU_0866	MEMBRANE
			Human	119	79	198			
			Mouse	7	1	8			
9	**DKEIRAISDYVVNHK**	**AAO90441.1**	**Cow**	**271**	**21**	**292**	***prpD***	**CBU_0912**	**CYTOPLASM**
			Human	**171**	**35**	**206**			
			Mouse	**6**	**1**	**7**			
235	RADMFIAVHADAYKN	AAO90598.2	Cow	250	42	292		CBU_1085	CYTOPLASM
			Human	84	101	185			
			Mouse	4	4	8			
581	DIPFHAVEIEKLAHR	AAO90739.1	Cow	286	7	293		CBU_1230	CYTOPLASM
			Human	157	36	193			
			Mouse	7	1	8			
255	DSDFIAFLKNQAADE	AAO90852.2	Cow	278	15	293		CBU_1349	CYTOPLASM
			Human	121	60	181			
			Mouse	5	2	7			
306	DNGYRSIHTAVIGPE	AAO90878.1	Cow	278	15	293	*relA*	CBU_1375	CYTOPLASM
			Human	190	15	205			
			Mouse	8	0	8			

205	**QGEYIIDIAEALKAK**	**AAO91497.1**	**Cow**	**267**	**28**	**295**	***argS***	**CBU_2008**	**CYTOPLASM**
			Human	**145**	**61**	**206**			
			Mouse	**5**	**3**	**8**			
252	DSYLKYAPIHAVGAP	AAO91502.1	Cow	292	3	295		CBU_2013	CYTOPLASM
			Human	137	66	203			
			Mouse	8	0	8			
108	YDSIIKFAKANKLRI	ACI15237.1	Cow	286	9	295		CBU_0095a	CYTOPLASM
			Human	153	52	205			
			Mouse	7	1	8			
Peptides binding all human MHC Class II alleles									
226	EGAIRHTHVIPIAGD	AAO89704.2	Goat	24	3	27	*ftsA*	CBU_0140	CYTOPLASM
			Sheep	20	1	21			
			Cow	280	15	295			
			Mouse	7	1	8			
259	QIKIKYASVLPEEVN	AAO89704.2	Goat	25	2	27	*ftsA*	CBU_0140	CYTOPLASM
			Sheep	19	2	21			
			Cow	273	22	295			
			Mouse	7	1	8			
9	**DKEIRAISDYVVNHK**	**AAO90441.1**	**Goat**	**27**	**0**	**27**	***prpD***	**CBU_0912**	**CYTOPLASM**
			Sheep	**21**	**0**	**21**			
			Cow	**271**	**21**	**292**			
			Mouse	**6**	**1**	**7**			
169	RQSIRYYHTAAAIKN	AAO90977.1	Goat	19	8	27		CBU_1480	Membrane
			Sheep	17	4	21			
			Cow	213	80	293			
			Mouse	7	1	8			
390	RGKFKIYIADPAIAP	AAO91005.1	Goat	25	2	27		CBU_1508	CYTOPLASM
			Sheep	21	0	21			
			Cow	232	59	291			
			Mouse	6	2	8			
678	GNKIIQIAPARVANR	AAO91357.1	Goat	25	2	27	*parC*	CBU_1866	CYTOPLASM
			Sheep	17	4	21			
			Cow	273	22	295			
			Mouse	7	1	8			
403	RQNIRAVDTQQVTAA	AAO91392.1	Goat	18	9	27		CBU_1901	Secreted
			Sheep	14	7	21			
			Cow	206	87	293			
			Mouse	7	1	8			

329	NNAIRYAKNVNVRIQ	AAO91494.1	Goat	25	2	27	*rstB*	CBU_2005	Membrane
			Sheep	21	0	21			
			Cow	283	12	295			
			Mouse	6	2	8			
205	**QGEYIIDIAEALKAK**	**AAO91497.1**	**Goat**	**27**	**0**	**27**	***argS***	**CBU_2008**	**CYTOPLASM**
			Sheep	**21**	**0**	**21**			
			Cow	**267**	**28**	**295**			
			Mouse	**5**	**3**	**8**			

T-cell epitopes predicted to bind all small ruminant alleles are above and epitopes predicted to bind all human alleles are below. The number of alleles each peptide interacted within other species either strongly (SB), weakly (WB), or in totality (TB) are notated. Bolded rows indicate that the predicted peptide interacted with all small ruminant and human alleles tested and underlined rows indicate peptides previously returned in Table 2. GenbankID, gene name, and locus tag are defined by NCBI. Inmembrane was used to predict protein location, where a potentially surface exposed protein is lowercase. Position and peptide are outputs from the NetBoIaIIpan bioinformatic program.

T-cell epitopes predicted for MHC Class I alleles of sheep and goats

Unlike the MHC Class II mature molecule using two proteins to produce the peptide binding groove, the MHC Class I peptide binding groove is only made from one protein [26]. For MHC Class I T-cell epitope prediction, the NetMHCpan program was used [28]. Similar to NetBoLAIIpan, the program was built initially to predict T-cell epitopes in the cattle species. Therefore, 160,000 randomly generated peptides were tested for each allele run on the program to generate a rank score to enable *C. burnetii* proteome analysis.

NetMHCpan breaks each protein into consecutive 8-, 9-, 10-, and 11-mers producing 1,196,551 peptides for testing from the conserved 1,002 *C. burnetii* proteins. Following loading of bioinformatic results into the SSMS database, it was calculated that 9,581 and 12,669 peptides for sheep and goat respectively reached the threshold of interacting with 60% of the species alleles tested or interacting strongly with 45% of the species alleles tested. Initial analysis of this data determined that 14 peptides were predicted to interact with all 56 sheep alleles strongly and that 25 peptides were predicted to interact with all 18 goat alleles strongly (**Table 4**).

Table 4. Pan-allelic peptides predicted to interact strongly with MHC Class I alleles tested for small ruminants.

Position	Peptide	GenbankID	Gene Name	Locus Tag	Location
Predicted strong binding pan-allelic peptides predicted for sheep MHC Class I presentation (56 alleles tested)					
302	**VTYPPPKTL**	**AAO89834.2**		**CBU_0276**	**CYTOPLASM**
333	RAYDVSFSL	AAO89886.2	*thiC*	CBU_0330	CYTOPLASM
172	**SSYPHPFLM**	**AAO90011.1**	***pdhA***	**CBU_0461**	**CYTOPLASM**
242	TVYPKTHYV	AAO90064.1	*uvrB*	CBU_0518	CYTOPLASM
114	**RAYEAIQSL**	**AAO90172.1**	***ppa***	**CBU_0628**	**CYTOPLASM**
67	**AAYTSPHLL**	**AAO90423.1**	***folC***	**CBU_0894**	**CYTOPLASM**
196	SVYEQVHLL	AAO90423.1	*folC*	CBU_0894	CYTOPLASM
302	YTFEKQLVL	AAO90655.1	*secD*	CBU_1142	Membrane
482	**SVYEGIPHL**	**AAO90700.2**	***ftsK***	**CBU_1191**	**Membrane**
335	FTYPKVPNL	AAO90866.1		CBU_1363	CYTOPLASM
126	SAFEWHLTF	AAO90883.1		CBU_1380	MEMBRANE
377	FSSPIFHEF	AAO91209.1		CBU_1714	CYTOPLASM
54	HTFPGVIQL	AAO91456.1		CBU_1967	MEMBRANE
64	IAAPLPIQL	ACI15273.1		CBU_1067a	Membrane

Predicted strong binding pan-allelic peptides for goat MHC Class I presentation (18 alleles tested)					
273	IQYQGPILL	AAO89579.1	*dacB*	CBU_0009	Secreted
6	MQVPLQITL	AAO89590.2		CBU_0020	CYTOPLASM
136	YQSEVQKEL	AAO89696.1	*ftsW*	CBU_0132	MEMBRANE
33	AQSPLLHYL	AAO89719.1	*pilB*	CBU_0155	CYTOPLASM
132	FQIKPPHQL	AAO89740.2		CBU_0180	Cell Wall
302	**VTYPPPKTL**	**AAO89834.2**		**CBU_0276**	**CYTOPLASM**
101	YQYDNVRSV	AAO89868.2		CBU_0311	Membrane
18	AQYPSPQLM	AAO89889.1	*thiG*	CBU_0333	CYTOPLASM
114	YQIELIIEL	AAO89932.1	*ampD*	CBU_0379	Cell Wall
119	KQADIYPTL	AAO89970.1		CBU_0418	CYTOPLASM
172	**SSYPHPFLM**	**AAO90011.1**	***pdhA***	**CBU_0461**	**CYTOPLASM**
39	QQLEPSVTL	AAO90063.2	*aspB*	CBU_0517	CYTOPLASM
220	YQKERVLTF	AAO90095.2	*rodA*	CBU_0549	MEMBRANE
10	TQFEDLPSL	AAO90111.1		CBU_0567	CYTOPLASM
114	**RAYEAIQSL**	**AAO90172.1**	***ppa***	**CBU_0628**	**CYTOPLASM**
77	FQFTRPHYL	AAO90288.1		CBU_0748	Lipoprotein
67	**AAYTSPHLL**	**AAO90423.1**	***folC***	**CBU_0894**	**CYTOPLASM**
126	SQLPVIQKL	AAO90606.1		CBU_1093	MEMBRANE
166	HQNTPIQQL	AAO90644.1		CBU_1131	CYTOPLASM
482	**SVYEGIPHL**	**AAO90700.2**	***ftsK***	**CBU_1191**	**Membrane**
62	FQLEHAHFL	AAO90820.1		CBU_1316	CYTOPLASM
513	SQQEKTIQL	AAO91182.1		CBU_1686	CYTOPLASM
156	SQNPALHAL	AAO91229.2		CBU_1735	Membrane
252	SSAPHTHAL	AAO91476.1	*apaH*	CBU_1987	CYTOPLASM
38	YQFPQAPNM	AAO91509.2		CBU_2021	CYTOPLASM

Pan-allelic strong scoring peptide interactions for sheep are above and those for goats are below. Bolded and underlined rows indicate predicted T-cell epitopes that remained the same between these small ruminant species. Position and peptide are generated by NetMHCpan, where the Genbank ID, gene name, and locus tag are from NCBI. Location is estimated by the Inmembrane program; lowercase locations indicate the potential for surface exposure.

Prediction of *C. burnetii* proteins which interact with cattle MHC Class I alleles has been presented previously [29]. Therefore, high scoring predicted T-cell epitopes were compared between all three ruminant species. Initially, this resulted in return of 3,547 peptides of interest, where this number dropped to 3,357 peptides when those overlapping by fifty percent, or more, were removed (**Supplemental Table 8**). Further filtering the data to isolate predicted T-cell epitopes that either bound all small ruminant alleles strongly or interacted with 98% of cattle alleles returned one peptide of interest. This peptide is present in **Table 4** for locus tag CBU_0628 at position 114.

MHC Class I T-cell epitopes predicted in all five species

Similar to cattle MHC Class I allele T-cell epitope prediction, mouse and human prediction for MHC Class I alleles has been published previously [29]. Comparison of high scoring T-cell epitopes between all five species (goat, sheep, cattle, human, and mouse) returned 766 predicted T-cell epitopes (**Supplemental Table 9**). As done for MHC Class II predicted T-cell epitopes, primary attention was given to the small ruminant and human species. When assessing for peptides predicted to strongly bind with all the small ruminant alleles tested, there were no additions or subtraction from the

bolded peptides in **Table 4**. Prior isolation of predicted T-cell epitopes that interacted with 90% of the tested human alleles, determined that there were 3 peptides of interest [29]. Of these, two peptides recurred in the present study (**Table 5**). The scores of the peptides as they relate to the other species tested are also present within **Table 5**, where the predicted T-cell epitopes are near the cut-offs given for exceptionally high interaction within the alternate species tested.

T-cell epitope dense proteins

Epitope dense proteins have been previously defined as proteins which contain multiple peptides of interest [29,70,71]. Prior evaluation of epitope dense proteins from *C. burnetii* was completed when assessing human, mouse, and cattle species [29]. Re-examination of the data following the addition of goat and sheep species resulted in a list of predominantly the same proteins of interest (**Supplemental Table 10**). Importantly there were 14 MHC Class II proteins and 3 MHC Class I proteins which no longer achieved the set cut-off values. Furthermore, there was one novel protein for MHC Class II, AAO90920.1 or *radA*, returned.

Comparison of MHC Class I and Class II predicted T-cell epitopes

In analyzing both MHC Class I and Class II allele types for predicted T-cell epitopes, we are able to evaluate for epitope dense proteins and for overlapping epitopes that fulfill both classes of MHC allele. Resultant data from all 5 species with overlapping peptides removed were combined for MHC Class II and MHC Class I. Afterwards, any protein suggested to contain at least two T-cell epitopes were isolated for further examination (**Supplemental Table 11**).

Recent experimentation indicated that surface isolated proteins from Nine Mile Phase II bacteria produced a protective immune response in mice and guinea pigs [72]. Furthermore, these isolated proteins lowered the clinical disease seen in non-human primates, where vaccinated animals did not become febrile or have an increased respiratory rate [72]. This data place emphasis on surface localized proteins; therefore, membrane localized or secreted proteins were isolated and are presented in **Table 6**.

Table 5. Exceptionally high scoring T-cell epitopes predicted for human MHC Class I.

Position	Peptide	GenbankID	Species	SB	WB	TB	Gene Name	Locus Tag	Location
54	HTFPGVIQL	AAO91456.1	Sheep	56	0	56		CBU_1967	MEMBRANE
			Mouse	3	4	7			
			Goat	17	1	18			
			Cow	75	26	101			
113	ATYGHIHQM	AAO91555.1	Sheep	54	2	56		CBU_2071	MEMBRANE
			Mouse	5	2	7			
			Goat	17	1	18			
			Cow	87	15	102			

GenbankID, gene name, and locus tag are as notated on NCBI under the Nine Mile RSA493 assembly. Position and peptide are designated by NetMHCpan, while location was previously predicted by Inmembrane. Species tested beyond humans have their scores for each peptide of interest. The calculated scores are for strong binders (SB), weak binders (WB), and total binders (TB).

Table 6. Overlapping and epitope dense membrane and secreted proteins predicted for all five species examined.

Genbank ID	MHC Class I	MHC Class II	Epitope Dense	Gene Name	Locus Tag	Location
AAO89616.1*	312	257, 338	No		CBU_0049	Membrane
AAO89682.2*	348	426, 508	No	*ftsI*	CBU_0118	Membrane
AAO89683.2	175	342	No		CBU_0119	Membrane
AAO89702.1*	19	214	No	*ftsQ*	CBU_0138	Membrane
AAO89725.1*	87	152	No		CBU_0165	Membrane
AAO89757.1	96, 147, 165, 375, 637, 755	748	Yes		CBU_0197	Membrane
AAO89774.2	38, 60, 355, 421, 488, 509	283	Yes		CBU_0215	Cellwall
AAO89870.2*	59	400	No		CBU_0313	Cellwall

AAO89926.1*	146, 291	302	No		CBU_0372	Membrane
AAO89978.2*	26, 211	59	No		CBU_0426	MEMBRANE
AAO90003.2*	3	49	No	*fimT*	CBU_0453	Membrane
AAO90031.1	49	246	No		CBU_0482	Secreted
AAO90110.1*	199	320	No		CBU_0566	MEMBRANE
AAO90155.1	470, 551, 795	257, 320	Yes	*yaeT*	CBU_0611	Membrane
AAO90276.1*	173	89	No		CBU_0736	Secreted
AAO90399.1*	63, 152	197	No		CBU_0866	MEMBRANE
AAO90424.2*	47, 132	190	No	*dedD*	CBU_0895	Membrane
AAO90487.1*	182, 304, 325	408	No	*cydA-2*	CBU_0965	Membrane
AAO90508.2*	184	62	No		CBU_0987	Lipoprotein
AAO90522.1*	265	396	No	*lolC*	CBU_1001	MEMBRANE
AAO90656.1*	81	8	No	*yajC*	CBU_1143	MEMBRANE
AAO90684.1	26, 222	208	No		CBU_1175	MEMBRANE
AAO90696.1	59	48, 197	No		CBU_1187	Secreted
AAO90700.2*	16, 288, 482	244	No	*ftsK*	CBU_1191	Membrane
AAO90703.1*	158, 241	166	No		CBU_1194	Membrane
AAO90737.1	150, 257, 419, 468	254	Yes	*qseC*	CBU_1228	Membrane
AAO90753.1*	117, 378, 431	427	No		CBU_1244	MEMBRANE
AAO90948.1*	294	73	No		CBU_1451	Membrane
AAO90965.2	109, 236, 367, 952	168, 608, 699	Yes		CBU_1468	Membrane
AAO90977.1*	8	169	No		CBU_1480	Membrane
AAO91047.2	236, 462	721	No	*ptsP*	CBU_1550	Membrane
AAO91053.1*	107	90	No		CBU_1556	MEMBRANE
AAO91093.1*	334, 538	666	No	*rpoD*	CBU_1596	Secreted
AAO91122.1*	179	18	No	*icmG*	CBU_1626	Membrane
AAO91140.1*	10, 93	192	No	*dotC*	CBU_1644	Lipoprotein
AAO91155.2	66, 97, 182	59	No		CBU_1659	MEMBRANE
AAO91229.2	44, 156	31	No		CBU_1735	Membrane
AAO91235.1*	39, 81	255	No		CBU_1741	Lipoprotein
AAO91260.1*	509	254	No	*feoB*	CBU_1766	Membrane
AAO91303.1	41	34, 85	No	*macA*	CBU_1810	Lipoprotein
AAO91311.1*	293	317	No		CBU_1818	MEMBRANE
AAO91329.1*	57, 285	40	No		CBU_1836	Membrane
AAO91360.1*	17	166	No		CBU_1869	Secreted
AAO91378.1	540, 553	103, 253	No	*ponA*	CBU_1887	Membrane
AAO91393.1	105, 117, 287	365, 409	Yes		CBU_1902	Secreted
AAO91411.1*	400, 532	185, 282	No	*yidC*	CBU_1920	MEMBRANE

AAO91419.2	11, 298, 505, 668	594	Yes		CBU_1928	Membrane
AAO91467.1	164, 171, 616, 833	730	Yes	*ostA*	CBU_1978	Secreted
AAO91474.1	125	121	No		CBU_1985	Membrane
AAO91516.1*	92	16	No		CBU_2029	Secreted
AAO91543.1*	320	139	No		CBU_2058	MEMBRANE

GenbankID, gene name, and locus tag are protein information isolated from NCBI from Nine Mile RSA493. MHC Class I and MHC Class II columns indicate the positions which are considered high scoring epitopes in all 5 species tested. A protein is considered epitope dense if it has greater than or equal to 5 predicted T-cell epitopes. Locations were previously predicted by the Inmembrane program, where a potentially surface exposed protein is notated by upper and lower case listed positions. Asterisks following the GenbankID denote newly predicted proteins of interest between this manuscript and the prior analysis completed with human, mice, and cattle only [29]. Overlapping MHC Class I and Class II epitopes have their positions underlined in their respective columns.

Filtering the data in this way returned 51 proteins of interest. Notably, 33 of these proteins are newly identified as compared to the previous study predicting T-cell epitopes in humans, mouse, and cattle species, but lacked small ruminant examination [29]. This emphasizes the importance of including all species that are rational choices for developing a subunit vaccine. Within the present proteins, there are 10 which contain T-cell epitopes predicted to overlap between MHC Class I and MHC Class II surface molecules. These include CBU_0197, CBU_1175, CBU_1187, CBU_1194, CBU_1228 (*qseC*), CBU_1244, CBU_1659, CBU_1735, CBU_1810 (*macA*), and CBU_1985. Furthermore, it is intriguing to observe that only 19 of the proteins within this list contain gene names. And while some of these proteins may have been studied by direct means, it is more than likely that homology between proteins of alternate bacterial species has allowed for the identification of the presently designated genes.

Discussion

T-cell epitope prediction has expanded in the last few decades to include species beyond humans and common animal models, mice and swine for example [27,28]. This is beneficial when studying animal health related pathogens or pathogens that are responsible for zoonotic transmission. *C. burnetii* is one such pathogen that can result in Q fever during human exposure and may lead to reproductive related issues in ruminant species [11,12]. The present study employed current bioinformatic T-cell prediction programs to evaluate T-cell epitopes for three ruminant species. This required isolation of sheep and goat alleles, execution of bioinformatic programs, and assembling resultant data in a comprehensive fashion. A summary of the additional documents is available in **Supplemental Table 12** to help the reader with file orientation.

After assessing the sheep and goat MHC alleles available for analysis, it was determined that the sheep species had a greater capacity to represent common North American breeds of interest. Notably, the IPD-MHC database maintains several sheep MHC alleles belonging to either Class I or Class II. Importantly, while many of the MHC Class II alleles have been directly sequenced from breeds of interest, the MHC Class I alleles have not been studied in depth. For this reason, whole genome sequences for sheep breeds of interest were isolated from NCBI and screened for additional MHC Class I sheep candidates. This increased the sheep MHC Class I alleles by twenty-four, of which both IPD-MHC and whole genome defined alleles were included in the present analysis. In contrast, assessing goat alleles present on the IPD-MHC database determined that further study is required to create an in-depth library of carried genes beyond the Scottish Cashmere breed [37]. Specifically, Ballingall et al. have presently sequenced twenty-eight MHC Class II alleles, of which two were identified when screening the Saanen and San Clemente genomes available on NCBI. Furthermore, there are no MHC Class I alleles at present for goat species on the IPD-MHC database and manuscript investigation only yielded one result [39]. Screening of the Saanen and San Clemente genomes produced seventeen MHC Class I alleles for testing in the present assay, but goat MHCs clearly require further definition.

Following allele definition, T-cell epitope predictions were run for the ruminant species and compared within the Bovidae family or between all five species of interest. The importance of interspecies comparison is demonstrated within recent *C. burnetii* research defining T-cell epitopes through T-cell recall responses against pathogen peptides [73-75]. Specifically, it was shown that several peptides could elicit IFN-γ secretion by human T-cells following natural exposure to *C. burnetii* during the 2007-2010 Netherlands outbreak [73,74]. A subset of the peptides which elicited a recall response were further tested within the mouse species, where it was determined that mouse T-cells had a recall response to only 33% of the tested peptides following *C. burnetii* inoculation [75]. Further testing within these experiments showed that mice predominantly responded to one of the twenty-seven tested peptides and that this response did not elicit protection during inoculation assays. A similar phenomenon can be seen herein, where 83 out of the 122 resultant proteins of interest returned in the present

study were novel compared to the prior study (**Supplemental Table 11**) [29]. While these numbers represent the initial assessment of high scoring proteins of interest, the pattern continued when considering MHC Class-overlapping T-cell epitopes and epitope dense proteins of interest. Specifically, when assessing peptides of interest that overlapped between the MHC Class I and Class II allele presentation, there were two new proteins of interest, CBU_1194 and CBU_1244. Accompanying these two additional proteins, there were fifteen proteins no longer present from the original dataset (**Supplemental Table 13**) [29].

Due to the interspecies differences seen, testing of the present T-cell epitope predictions needs to be indicated by vaccine direction. Efforts to employ common *C. burnetii* proteins in vaccine development studies have occurred; however, subunit vaccine research has typically been focused on human health employing mouse models [73-76]. While safety and cost concerns continue to prohibit mass production of a formalin-inactivated whole cell vaccine within the United States, studies assessing these bacterins in other countries have permitted researchers a glimpse of protective phenotypes [15,17,77,78]. Future studies could choose to investigate proteins that are considered epitope dense or peptides which are suggested suitable to both MHC Class I and Class II presentation. While the current manuscript has not eliminated predicted epitopes of interest based on these distinctions, it has primarily focused on surface localized or secreted proteins. This is due to work by Gregory et al., which described surface isolated Nine Mile phase II proteins producing a protective immune response in both guinea pigs and mice [72].

It is important to mention that prior work promoting generation of a *C. burnetii* vaccine has suggested that multiple epitopes are required to produce a protective immune response [75,79-82]. For this reason, scientists have generated preliminary vaccines containing up to 28 epitopes of interest [75]. Keeping with this rationale would promote testing of overlapping MHC Class I and Class II T-cell epitopes, such as the 10 epitopes found in **Table 6**. Notably, only two of these proteins have a given name, MacA and QseC. Studies on either protein demonstrate the correct cellular location by Inmembrane, as they both contain transmembrane and periplasmic domains [35,83-86]. Direct study of the *C. burnetii* derived proteins has not yet occurred, but there is research from other Gram-negatives, like *Escherichia coli*. Specifically, MacA serves in a multimeric complex shown to export macrolides from inside the bacterium to the external environment [83,86]. In contrast, QseC is part of the quorum sensing cascade that can result in alternate transcription of virulence factors or motility components [84,85]. As can be seen, either protein's activity is critical for bacterial virulence. Of the remaining eight overlapping MHC Class I and Class II epitopes, there are two new within this study due to the inclusion of small ruminants; comprised of CBU_1194 and CBU_1244. Even though these ten proteins represent a small subset of the overall proteome, there are a number of annotated hypothetical proteins which would benefit from further characterization. For example, recent examination of CBU_0307, which was previously described as containing a high scoring MHC Class I human T-cell epitope, has discerned the protein's importance in membrane structural integrity [29,73,87].

Conclusion

Overall, this manuscript expands the scope of comparative predicted *C. burnetii* T-cell epitopes to include the small ruminant species. This represents one of the few manuscripts which has accounted for the cross-species *C. burnetii* pathogenesis during T-cell epitope prediction. During this analysis, it was determined that MHC allele characterization is required for the goat species. Nevertheless, novel proteins of interest were identified when considering the additional goat and sheep species. While T-cell epitopes of interest have been predicted, their ability to stimulate T-cells will be further elucidated by future work.

Declarations

Availability of data and materials

The previous dataset from the manuscript titled "Proteome-wide analysis of *Coxiella burnetii* for conserved T-cell epitopes with presentation across multiple host species," is available on an Open Science Framework repository with accession number RN6QA (https://osf.io/rn6qa/). The present study has analyzed data within the published article, supplementary information, and a separate online repository. The datasets generated by this study are available in the National Agricultural Library Ag Data Commons through the Figshare portal under the title "Data for: Expansion of Proteome-wide *C. burnetii* Comparative T-Cell Epitope Prediction to Include Small Ruminant Hosts" (https://doi.org/10.15482/USDA.ADC.24712827.v1).

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Authors' contributions

Conceptualization: LMWP, SNW; Methodology: LMWP, PCG, RK, DAS; Formal Analysis: LMWP; Writing-Original Draft: LMWP, PCG; Final Writing & Editing: LMWP, PCG, DAS, RK, SNW; Data curation: LMWP, RK, DAS. All authors read and approved the final manuscript.

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