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# *Mannheimia haemolytica* IgA-specific proteases

Sahlu Ayalew, Betsy K. Murdock, Timothy A. Snider, Anthony W. Confer*

*Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK, 74078-2007, USA*

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ABSTRACT

*Mannheimia haemolytica* colonizes the nasopharynx of cattle and can cause severe fibrinous pleuropneumonia. IgA proteases are metalloendopeptidases released by bacteria that cleave IgA, enhancing colonization of mucosa. The objectives of these studies were to characterize *M. haemolytica* IgA1 and IgA2 proteases *in vitro* and *in silico*, to clone and sequence the genes for these proteases, and to demonstrate immunogenicity of components of the entire IgA protease molecule. Both IgA protease genes were cloned, expressed, and sequenced. Sequences were compared to other published sequences. Components were used to immunize mice to determine immunogenicity. Sera from healthy cattle and cattle that recovered from respiratory disease were examined for antibodies to IgA proteases. In order to assay the cleavage of bovine IgA with IgA1 protease, *M. haemolytica* culture supernatant was incubated with bovine IgA. Culture supernatant cleaved purified bovine IgA in the presence of $ZnCl_2$. Both IgA proteases contain three domains, 1) IgA peptidase, 2) PL1_Passenger_AT and 3) autotransporter. IgA1 and IgA2 peptidases have molecular weights of 96.5 and 87 kDa, respectively. Convalescent bovine sera with naturally high anti-*M. haemolytica* antibody titers had high antibodies against all IgA1 & IgA2 protease components. Mouse immunizations indicated high antibodies to the IgA peptidases and autotransporters but not to PL1_Passenger_AT. These data indicate that *M. haemolytica* produces two IgA proteases that are immunogenic, can cleave bovine IgA, and are produced *in vivo*, as evidenced by antibodies in convalescent bovine sera. Further studies could focus on IgA protease importance in pathogenesis and immunity.

## 1. Introduction

Most Gram-negative pathogens have adapted ways to maintain colonization of mucosal surfaces. Several bacterial strategies for colonization are found. These include adherence through numerous adhesins, evasion of host immunity often through secretion of toxins and proteases, and production of capsular polysaccharides and biofilm (Miajlovic and Smith, 2014). To assist in evasion of host mucosal immunity, numerous pathogenic bacteria have acquired mechanisms to subvert the antibacterial actions of mucosal IgA. Key among those mechanisms is production of highly specific IgA-cleaving proteases (Diebel et al., 2009; Kilian et al., 1996). Bacterial IgA proteases are autotransporter molecules, which are conserved proteins synthesized as large precursors with a C-terminus autotransporter, a translocator domain, and one or more passenger peptidases that are the functional secreted domains of the protein (Diebel et al., 2004; Klauser et al., 1993; Mistry and Stockley, 2006; Parsons et al., 2004). IgA peptidases constitute the passenger component of many autotransporter proteins. Pathogenic bacteria producing IgA proteases include *Escherichia coli, Burkholderia spp., Streptococcus pneumoniae, Neisseria spp., Haemophilus influenzae, and Haemophilus parasuis.* (Binscheck et al., 1995; Clementi et al., 2014; Gilbert et al., 1988; Janoff et al., 2014; Lazar Adler et al., 2011; Mullins et al., 2011) Gram-negative IgA proteases are genetically similar; for example, *H. influenzae* and *Neisseria spp.* IgA1 proteases are approximately 52% identical (Parsons et al., 2004). IgA proteases cleave IgA into Fc and Fab components, reducing the ability of IgA to inhibit bacterial events, because antibodies can no longer link Fab binding with Fc-mediated elimination mechanisms such as opsonization or complement-mediated killing (Woof and Kerr, 2006). In several bacteria, IgA protease secretion is associated with disease-causing strains, whereas IgA protease-deficient strains are often non-pathogenic (Knapp et al., 1984) Several bacterial strains contain two distinct IgA proteases, and invasion of respiratory cells by nontypeable *H. influenzae* is enhanced in bacteria that produce both IgA proteases (Kostyanev and Sechanova, 2012). In addition to its IgA protease inhibitory function, IgA proteases can stimulate release of proinflammatory cytokines TNFα, IL-6, and IL-8 while not inducing regulatory cytokine IL-10 (Lorenzen et al., 1999). Because of their function and surface exposure of certain domains and secreted proteases, autotransporter proteins are virulence factors that can serve as potential targets for immunity (Wells et al., 2007).

*Mannheimia haemolytica* causes severe, often fatal, pneumonia in cattle, especially in Shipping Fever in weaned beef cattle (Panciera and

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* Corresponding author at: Department of Veterinary Pathobiology, 250 McElroy Hall, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, OK, 74078-2007, USA.

*E-mail address:* anthony.confer@okstate.edu (A.W. Confer).

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Confer, 2010). The bacterium is a natural inhabitant of the upper respiratory tract of cattle (Frank and Briggs, 1992). Multiple surface adhesins and biofilm formation enhance nasal and nasopharyngeal colonization (Boukahil and Czuprynski, 2016; Confer, 2009; Gioia et al., 2006; Kisiela and Czuprynski, 2009). Stress due to environmental changes, shipping, weaning, comingling, and viral infections cause the bacterium to proliferate, to release from the nasopharyngeal surface, and to be inhaled into the lower respiratory tract, often precipitating severe fibrinous bronchopneumonia and death (Pillai et al., 2018; Singh et al., 2011). Current control measures for bovine respiratory disease (BRD) include aggressive antibiotic therapy of sick animals, mass antibiotic treatment of all cattle upon arrival (metaphylaxis), and use of commercial and autogenous vaccines (Confer and Ayalew, 2019; Duff and Galyean, 2007). Commercial *M. haemolytica* vaccines are only partially efficacious in field trials (Larson and Step, 2012). Despite these measures, BRD remains the major cause of beef cattle morbidity, mortality, and reduced production, which costs the United States cattle industry about $1 billion per year (Miles, 2009).

Lee and Shewen (Lee and Shewen, 1996) identified an $IgG_1$-specific protease in partially purified culture supernatant from *M. haemolytica*. However, there has been no further characterization of that protease, and a gene for $IgG_1$ protease has not been described in the published *M. haemolytica* genomes (Ayalew 2018, unpublished data). The genes for IgA1 and IgA2 proteases were identified *in silico* in the *M. haemolytica* genome (Gioia et al., 2006; Harhay et al., 2013; Hauglund et al., 2013) and in the *M. haemolytica* secretome by proteomic analysis (Ayalew et al., 2017). The purposes of this study were to characterize *M. haemolytica* IgA proteases by *in silico* techniques, to clone and sequence the genes for these proteases, to demonstrate cleavage of bovine IgA, and to demonstrate immunogenicity of components of the entire IgA protease molecule. In this manuscript, we will refer to the entire molecule as an IgA protease and refer to the IgA-specific enzyme as IgA peptidase.

## 2. Materials and methods

### 2.1. Identification and description of IgA proteases

*Mannheimia haemolytica* serotype S1, strain 89010807 N, originally isolated from a case of calf pneumonia, was used for this study (Murphy et al., 1995). Growth conditions of the bacterium have been described (Ayalew et al., 2010). For identification of IgA proteases, *M. haemolytica* were grown to mid-log phase in Brain Heart Infusion broth, cells were removed by centrifugation at 10,000-x g for 10 min, and supernatant was filtered through 0.2 μm filters. Cell-free supernatants were concentrated with Centricon Plus-70 with MWCO of 3,000 (Millipore, Billerica, MA). Concentrated supernatants were submitted to Recombinant DNA/Protein Resources Facility of OSU where proteins in the supernatant were identified by LC-MS/MS and analyzed by proteomic analysis software such as PSORTb; SignalP4.1; SecretomeP; LipoP; TatP; Phobius SP and PRED-TAT (Ayalew, et al., 2017). Fresh or frozen concentrated culture supernatants of *M. haemolytica* were used as source of native IgA proteases.

Recombinant forms of IgA1 and IgA2 proteases were designed, cloned, expressed, and purified in our laboratory by using genomic DNA from *M. haemolytica* as templates and various primer pairs shown in Table 1. Twelve recombinant proteins were made and used in varieties of ways in this study. Each DNA encoding for the domain of interest in IgA proteases was obtained by PCR and ligated to expression vectors such as pET28a, b, or c. Following confirmation of the integrity by restriction enzyme analyses and sequencing, each was introduced into an appropriate expression host such as BL21(DE3), Rosetta 2 (DE3) where corresponding recombinant proteins were overexpressed and purified on HisTrap FF columns using ACTA purifier 10 (GE healthcare biosciences, Piscataway, NJ, USA) (Ayalew et al., 2011a). All recombinant proteins were purified under denaturing conditions (6 M urea) after which time the denaturant was reduced by stepwise dialysis to a level that did not result in the precipitation of the proteins. The names and brief descriptions of the recombinant proteins generated or developed in this work as shown in Table 2. With the exception of SAC222, the protocols are essentially identical. SAC222 was made by inserting a thrombin cleavage site close on the C-terminus of His-tag, which was subsequently removed using a thrombin cleavage capture kit (Millipore Sigma, Temecula, CA). Purified recombinant proteins and supernatants of each expression were concentrated and used in assays.

### 2.2. Source of bovine IgA

Bovine IgA was purified from fresh bovine colostrum as described (Nielsen, 1986). After separating whey and casein by acidification to pH 4.2 with glacial acetic acid and removing the curd by filtration, bovine IgA was purified on HiPrep Sephacryl 100 HR (GE HealthCare, Piscataway, NJ) column by size exclusion chromatography. Fractions containing IgA were identified by Western blot analysis using anti-bovine IgA antibodies and pooled for use in IgA cleavage assays.

### 2.3. IgA peptidase activity assays

To determine IgA peptidase activity, 5 μg of purified bovine IgA and 6 μg of *M. haemolytica* culture supernatants were mixed in 50 mM Tris-HCl, pH 7.5, and 5 mM of $CoCl_2$ or $MnCl_2$, or $ZnCl_2$ in a total volume of 10 μl. Mixtures were incubated in a 37 °C water bath for 2 h. Equal volume of 2X gel loading buffer was added to each tube, and the tubes were placed in boiling water bath for 5 min. Aliquots of the supernatant/IgA mixtures were resolved on a 4–15% SDS-PAGE at 50 V. Duplicate gels of each assay were run, one was stained with Coomassie brilliant blue, and the other was transferred onto nitrocellulose membranes and probed with 1:10,000 dilution of HRP-conjugated rabbit anti-bovine IgA antibody (Thermo Scientific, Rockford, IL) in a western blot. Membranes were incubated in SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) for 5 min and developed on X-ray films. Bands suspected to be cleaved IgA were cut from the Coomassie stained gel and sent for identification by LC-MS/MS. Functionality of rIgA1 and rIgA2 peptidases were determined similarly using 6 μg of recombinant proteins with 5 μg of bovine IgA.

### 2.4. Immunogenicity of recombinant IgA1 peptidase in mice

Mouse immunization studies were approved by the Oklahoma State University Institutional Animal Care and Use Committee (protocol #VM1045). Three experiments were conducted (A, B, and C), each using 50 female BALB/c mice weighing between 20–24 g (Charles River Laboratories, Wilmington, MA). Studies were conducted and mice were housed in the Animal Resources facilities of Oklahoma State University, an AAALAC-accredited facility. Mice were divided among three groups. For each study, Group 1 (n = 20) consisted of nonvaccinated controls. Groups 2 and 3 (n = 15 each) were vaccinated subcutaneously on days 0 and 14 with 50 or 5 μg of SAC 175 (rIgA1peptidase, Experiment A), SAC186 (rPL1_Passenger_AT, Experiment B), and SAC199 (rAutotransporter, Experiment C) in Freund's incomplete adjuvant, respectively. In each experiment, on day 0, 10 control mice were anesthetized, bled, and euthanized. On day 14, five mice each from Groups 2 and 3 were anesthetized, bled, and euthanized. On day 28, the remaining mice each from Groups 1–5 were anesthetized, bled, and euthanized and feces collected from each mouse. Sera were diluted 1:1600, and IgG, serum antibodies against rIgA1, rPL1_Passenger_AT, or rAutotransporter were determined by ELISA in 96-well microtiter plates coated with 0.5 μg/ml of SAC175. Details of the assay are as previously described (Ayalew et al., 2011b). Serology data are presented as mean $OD_{490}$ ± SEM.

### 2.5. Serum antibodies against IgA protease components in convalescent bovine sera

Forty bovine sera from weaned, crossbreed, beef cattle were used for this study. Twenty sera (Controls) were from calves with low serum

**Table 1**
Primers used for cloning of components of IgA proteases.

| Construct | Primers | Primer sequence | Recombinant Protein name |
|---|---|---|---|
| SAC163 | IgA ProteaseF<br>IgA ProteaseR | CTAGccatggTAAATAAAAACTTTCCACTTTCTTACC<br>AGCTctcgagCCAGCTATATCCCAGTTTGAAG (4488-4509) | Multi-domain IgA Protease |
| SAC164 | IgA Protease Ft<br>IgA Protease R | GATCccatggTTTTCAGAGATGATATCGATCTTCAATAC(79 -108):<br>AGCTctcgagCCAGCTATATCCCAGTTTGAAG (4488-4509) | Truncated IgA protease |
| SAC168 | IgA-Pep F<br>IgA-Pep R | TTATggatccGATGCGATCAATAAGCAAGATG (2281:2302)<br>ACGTctcgagTTATGGATAGTAACGGCGGTAGTAATCAG (3305:3328) | |
| SAC169 | IgA comp F<br>IgA R2 | GATCccatggTTCCGTAGCTGCTTTTGCCTATC<br>AGctcgagTTACCAGCTATATCCCAGTTTGAAG | 146 base pairs upstream – IgA with its promoter |
| SAC173 | IgA2 TR<br>IgA2 R | TAGCccatggCTGTTGTACGTAATGATGTGGACTAT<br>CGATctcgagCCAGCTATATCCTAGCTTAACACC | truncated IgA2 protease |
| SAC174 | IgA2 F<br>IgA2 R | ATACccatggATGAAGACCAAAACATTTACT<br>CGATctcgagCCAGCTATATCCTAGCTTAACACC | Full length IgA2 protease |
| SAC175 | IgA Protease F<br>IgA1 pro rev | CTAGccatggTAAATAAAAACTTTCCACTTTCTTACC<br>CTAGctcgagGCTATCCCCAGTGTTTGTCCAG | IgA1 peptidase |
| SAC176 | IgA2 TR<br>IgA2 pr rev | TAGCccatggCTGTTGTACGTAATGATGTGGACTAT<br>CTCGctcgagACCATTGTTTAACCAGTGACTGTGATTTG | IgA2 peptidase |
| SAC186 | Forward 186 F<br>Reverse 186 R | CAGCTTTATGccatggTTGGTCGTAATGTCTCAAACCT<br>CTTTAGCATCctcgagTTTTAACGGATTGTATAAGCGATAGC | PL1_Passenger_AT with C-terminus His-tag |
| SAC187 | Forward 187 F<br>Reverse 187 R | CAGCTTTATGccatggTTGGTCGTAATGTCTCAAACCT<br>CTTTAGCATCctcgagTTATTTTAACGGATTGTATAAGCGATAGC | PL1_Passenger_AT with N-terminus His tag |
| SAC199 | IgAaut F<br>IgAaut R | GGCGccatggAGATACCGACTCGTTTTAATA<br>GATcctcgagCCAGCTATATCCCAGTTTGAA | Autotransporter |
| SAC222 | | | Same as SAC175 but without His-tag |

**Table 2**
*M. haemolytica* IgA1 & IgA2 recombinant proteins.

| Construct: Identification | Co-ordinates |
|---|---|
| SAC163: Full length multi-domain IgA1 protease with C-terminus His Tag | 1-1503 |
| SAC164: Truncated SAC163 with signal peptide | 23-1503 |
| SAC167: SAC163 without His-tag | 1-1503 |
| SAC168: Short Internal region of IgA1 protease | 761-1110 |
| SAC169: SAC167 with promoter upstream of the ORF | 1-1503 |
| SAC173: Truncated IgA2 but same as SAC164 | 27-1374 |
| SAC174: IgA2 but same as SAC163 | 1-1398 |
| SAC175: IgA1 Peptidase, the enzyme alone, C-terminus His-tag | 27-899 |
| SAC176: IgA2 Peptidase, the enzyme alone, C-terminus His-tag | 27-815 |
| SAC186: PL1_Passenger_AT with C-terminus His-tag | 794-1030 |
| SAC187: PL1_Passenger_AT with N-terminus His-tag | 795-1030 |
| SAC199: Autotransporter | 923-1503 |

antibodies to *M. haemolytica* as determined by ELISA and with no history of respiratory disease, (Confer et al., 1997), and 20 sera were from cattle convalescent from naturally acquired shipping fever. Using ELISA, serum antibodies against rIgA1 peptidase (SAC175 or SAC222 i.e., the same as SAC175 except it lacks His-tag), rIgA2 (SAC176), rPL1_Passenger_AT (SAC186), and rAutotransporter (SAC199) were determined. ELISAs were done as previously described for other *M. haemolytica* recombinant proteins with the following modifications (Confer et al., 2003). Microtiter plate wells were coated with 50 ng of antigen. Bovine sera were diluted 1:800, with horseradish peroxidase-conjugated, rabbit anti-bovine IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD). Serum dilution is in the linear range of a dilution curve. Antibodies to formalin-killed *M. haemolytica* (whole cells) demonstrated the seronegative and convalescent nature of the two groups (Confer et al., 1997). Data are expressed as ng IgG binding based on a set of IgG standards on each plate.

### *2.6. Statistical analysis*

Mean serum antibody concentrations were compared between control and convalescent cattle groups using Student *t*-test (Petrie and Watson, 1999). Antibody responses among the mouse groups were compared by one-way analysis of variance with Tukey's HSD post hoc test. Differences were considered significant when $p < 0.05$.

## 3. Results

### *3.1. Cloning of components of M. haemolytica IgA proteases*

Genes for 12 different recombinant molecules were successfully cloned and expressed. These included the entire IgA1 and IgA2 proteases (Table 2). For functionality assays, SAC163 and SAC173 were used. For serology, SAC163, SAC173, SAC186, and SAC199 were used.

### *3.2. Physical map and sequence analysis of IgA proteases*

Overall comparison of the two proteases shows that with 1503 amino acids and a MW of 164.5 kDa, IgA1 protease is larger than IgA2 protease that has only 1398 amino acids long and a calculated MW of 155.6 kDa (Figs. 1 and 2). A deeper analysis of both IgA1 and IgA2 proteases showed that both proteins have three domains viz., IgA peptidase, PL1_Passenger_AT, and autotransporter on the C-terminus. The physical map of the three domains in IgA1 proteases is shown in Fig. 1, and their respective sequences are given in Supplemental Fig. 1). IgA peptidases of IgA1 protease (amino acids 29–899) with a MW of 95 kDa are bigger than IgA peptidases (amino acids 25–811) from IgA2 protease with a calculated MW of 87 kDa.. Likewise, PL1_Passenger_AT domain of IgA1 proteases with an average MW of 40.1 kDa (amino acids 795–1030) are comparable in size to similar domains (25.7 kDa and amino acids 712–746) in IgA2 proteases. In addition, autotransporters (amino acids 1139–1503) from IgA1 proteases with an average MW of 40.1 kDa are larger than autotransporters from IgA2 protease with an average MW of 20.5 kDa (amino acids 1146–1329).

Global amino acid sequence alignment of complete IgA1 and IgA2 proteases using ClustalW (MacVector version 17.0.5 software) and their respective domains from varieties of *Mannheimia haemolytica* strains had substantial similarities. These alignments are shown in Fig. 2 and sequences of the same are shown in Supplemental Fig. 2. Overall, a 53% match was observed in the total amino acid sequences of IgA1 and IgA2 proteases. Interestingly, IgA peptidases from both IgA proteases also had a 53% match in their amino acid sequences. Similarly, there were 55%, and 35% matches between PL1_Passenger_AT and autotransporter domains, respectively, of both IgA1 and IgA2 proteases. The autotransporters from IgA1 with 365 amino acids are larger than autotransporters from IgA2 proteases (184 amino acids).

*In silico* alignment comparing IgA1 protease gene sequences for *M. haemolytica* strain 89010807 N and published *M. haemolytica* S1 genomes

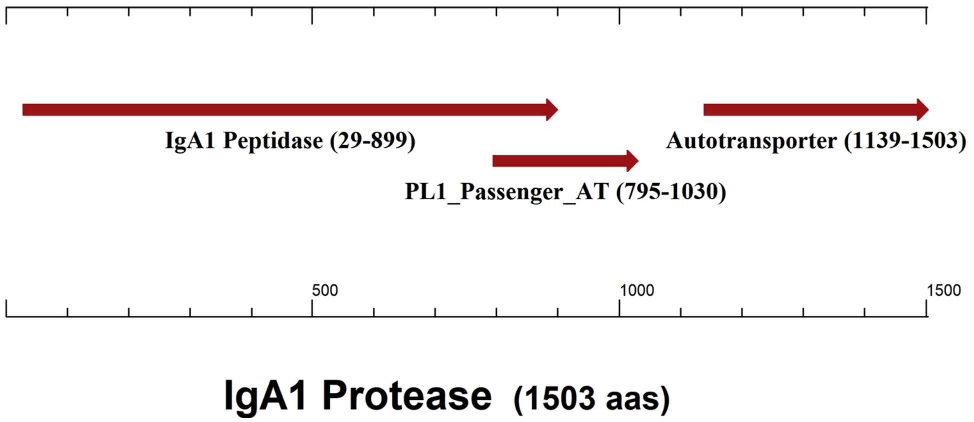

**Fig. 1.** Physical map of IgA1-specific protease (1503 amino acids) and the three domains comprising the whole protein viz., IgA1 peptidase, PL1_Passenger_AT and autotransporter regions. For detailed sequences of these regions, see Supplemental Fig. 1.

indicated identity among the 33 strains with very few exceptions (Supplemental Fig. 3). *M. haemolytica* IgA1 protease sequences from strains 89010807 N and 183 were aligned with IgA1 protease sequences from *H. influenzae* and *N. meningitidis,* and similarities were 29% and 30%, respectively, when considering the entire molecules. Further analyses by plotting amino acid Pustell Protein Matrices using MacVector ver. 17.0.5 revealed the existence of short imperfect repeats scattered all over IgA peptidase. There were, however, two direct repeats, 69 amino acids long at co-ordinates 505–573 (tingpkppeatdlkwgtwkensadiyeyinphannrtdyftlkgnpnqymptngasnahwtflssnkda) and 606–674 (rlnvvydpkastppatasevtwgkglvagadiylftnpktkireyfalkgdpkqpvprgglssehwefl). PL1_Passenger_AT had only a pair of imperfect repeats between 11–22 (vslaeqsqlhigka) and 65–78 (lsandhaqlhlgfi), and autotransporter had two pairs of imperfect repeats between 231–243 (ghnfdlagvqvqp) and 254–266 (aqnyklgevevrs) and 240–250 (qvqpavgtryy) and 290–300 (kvepslaahyv).

Similarly, alignment of IgA2 protease amino acids from 38 *M. haemolytica* S1 strains revealed that they are identical with rare exceptions indicating that IgA2 protease is also conserved among tested strains (Supplemental Fig. 4).

### 3.3. IgA protease cleavage of bovine IgA

As was described above, native IgA protease activity in *M. haemolytica* culture supernatants and recombinant forms of IgA1 and IgA2 peptidases were tested for their ability to cleave bovine IgA in the presence of metallic ions (Fig. 3). A cleavage product of approximately 40 kDa is seen in both Coomassie stained gel and its corresponding western blot (lane 5). Cleavage of bovine IgA requires the presence of $ZnCl_2$. Similarly, rIgA1-specific peptidase activity (SAC175 or SAC222) was demonstrated. Bovine IgA cleavage products in the presence of $ZnCl_2$ are similar to those seen with culture supernatant and $ZnCl_2$. No bovine IgA cleavage was observed when lysates or supernatants of Rosetta 2 (DE3) were assayed as described (Data not shown). LC-MS/MS tandem mass spectrometry was used to identify the cleavage products as components of bovine IgA.

### 3.4. Antibody responses of mice following rIgA1 peptidase, PL1_Passenger_AT, or autotransporter vaccination

Subcutaneous vaccination of mice with either 5 or 50 μg of SAC175 SAC186, or SAC199 resulted in significant increases ($p < 0.05$) in serum antibody responses against gA1 peptidase and autotransporter by 14 days after vaccination (Fig. 4). There were significant increases ($p < 0.05$) between days 14 and 28 for both vaccine groups, and sera from the 50-μg vaccine group had significantly higher antibodies at day 28 than did the 5-μg vaccine group. Serum antibodies against rPL1_Passenger_AT increased slightly for the 50-μg vaccinated group (0.33 ± 0.19) in Experiment B; however, those changes were not significantly greater ($p > 0.05$) than control values (0.20 ± 0.11) (data not shown).

### 3.5. Antibodies to components of IgA proteases in convalescent bovine sera

Serum antibodies against IgA1 peptidase, IgA2 peptidase, PL1_Passenger_AT, and autotransporter were significantly increased ($p < 0.01$) in convalescent cattle compared to healthy cattle that had no history of BRD (Table 3).

## 4. Discussion

It is well established that *M. haemolytica* readily colonizes nasal and nasopharyngeal mucosa. Mechanisms of colonization are not completely understood, but adhesins such as capsule, fimbria and outer membrane proteins have been identified (Kisiela and Czuprynski, 2009; Morck et al., 1988; Potter et al., 1988). Evasion of host immunity within the nasopharynx could be through several mechanisms. *M. haemolytica* exists on surfaces in a polysaccharide biofilm, which may assist in protecting the bacterium from host defenses (Boukahil and Czuprynski, 2016). The capsule allows the bacterium to resist phagocytosis and complement-mediated killing (Chae et al., 1990). Neuraminidase and sialoglycoprotease activities may also assist the bacterium in maintaining colonization (Lee et al., 1994; Straus and Purdy, 1995). We demonstrated by proteomic analyses a putative IgA protease in *M. haemolytica* culture supernatant, and in this manuscript demonstrated that the bacterium produces two IgA proteases that are organized similarly to those produced by other bacteria, i.e. IgA peptidase, PL1_Passenger_AT, and autotransporter (Ayalew et al., 2017). In addition, we demonstrated that IgA-specific protease activity is present in *M. haemolytica* supernatants, and IgA peptidase activity may assist the bacterium in evading IgA-mediated mucosal immunity.

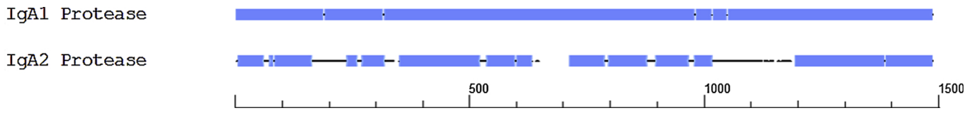

**Fig. 2.** Graphical comparison of the alignment similarities between genes from *M. haemolytica* strain 89010807 genes for IgA1 and IgA2 proteases. Areas in blue indicated significant similarity. Sequences of these aligned are shown in Supplemental Fig. 2.

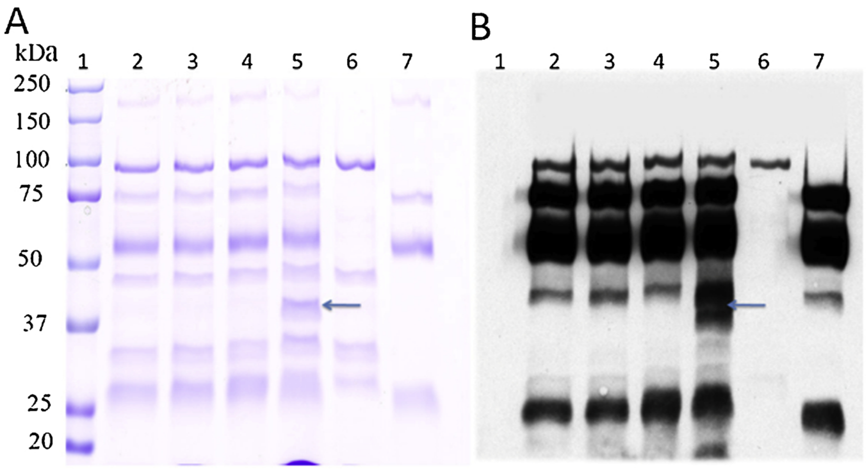

**Fig. 3.** Coomassie stained gel (A) and corresponding western blot using rabbit anti-bovine IgA antibody (B) showing M. haemolytica supernatant IgA-specific protease activity assay. Blue arrows indicate approximately 38 kDa cleaved portion of IgA due to IgA-specific protease activity in the presence of ZnCl2. Lane 1- Kaleidoscope Protein standards (Bio-Rad, Hercules, CA); 2 – Supernatant + IgA; 3 – Supernatant + IgA + CoCl2; 4 – Supernatant + IgA + MnCl2; 5 – Supernatant + IgA + ZnCl2; 6 – Supernatant alone and 7 – IgA alone.

*M. haemolytica* produces two IgA proteases; as with those produced by other bacterial species, IgA1 & IgA2 proteases are quite similar, especially the PL1_Passenger_AT and autotransporter regions. Sequences for those proteases were demonstrated within multiple published *M. haemolytica* sequences. Humans have two subclasses of IgA, IgA1 and IgA2. Bacterial IgA1 protease is so-named from human pathogens, because that protease only cleaves human IgA1 and not IgA2 (Senior et al., 2000). Cattle, however, have only one IgA type, and the naming of IgA1 and IgA2 proteases is based on sequence similarities between these two proteases and those produced by other bacteria (Tizard, 2004).

The recombinant forms of IgA1-peptidase and autotransporter were immunogenic in mice, whereas recombinant *M. haemolytica* PL1_Passenger_AT stimulated only low antibody responses that were not significantly different from control values. PL1_Passenger_AT from other bacteria, such as *Bordetella spp.*(pertactin), is an important immunogen (Kerr and Matthews, 2000). Antibodies against *M. haemolytica* IgA1 peptidase, IgA2 peptidase, PL1_Passenger_AT, and autotransporter were found in sera from BRD-convalescent cattle and were low in sera from cattle that had not been sick with BRD. Thus, during *M. haemolytica* infection in cattle, all components of the IgA proteases are immunogenic. Therefore, the inability to demonstrate antibody responses to PL1_Passenger_AT in mice may be due to the protocol or mouse strain used as well as the conformation of the recombinant protein.

The significance of the expression of IgA proteases *in vivo* demonstrated by presence of immune responses in convalescent sera is not known. It likely indicates that as *M. haemolytica* proliferates in cattle with BRD, the bacterium readily produces IgA proteases. Whether those IgA proteases are of pathogenic significance is not known. If the enzymes are expressed within the nasopharynx as the bacterium proliferates, they may counter anti-*M. haemolytica* IgA antibodies, as seen with other bacteria (Murphy et al., 2015). If, however, the IgA proteases are mainly expressed when the bacterium is replicating in the lung, their biological significance may be nil due to the insignificance of IgA antibodies at the lung level.

Because of their function as potential protectors of bacteria against mucosal immunity, IgA proteases have been proposed as immunogens for mucosal immunity (Fu et al., 2016; Kotel'nikova et al., 2014; Kotelnikova et al., 2016). In addition, epitopes of bacterial autotransporters are present on the surface of bacteria and are, therefore, potential targets for immunity (Ha et al., 2015; Suzuki et al., 2017; Wells et al., 2007). Future studies should consider if *M. haemolytica* IgA proteases could be components of a mucosal vaccine, and if so would neutralizing antibodies against IgA proteases enhance host resistance against the bacterium by preventing subversion of mucosal anti-*M. haemolytica* IgA antibodies by IgA protease (Kirkeby et al., 2000). In addition, small-molecule inhibitors of IgA1 protease are being studied for potential therapeutic use against *H. influenzae* (Shehaj et al., 2019).

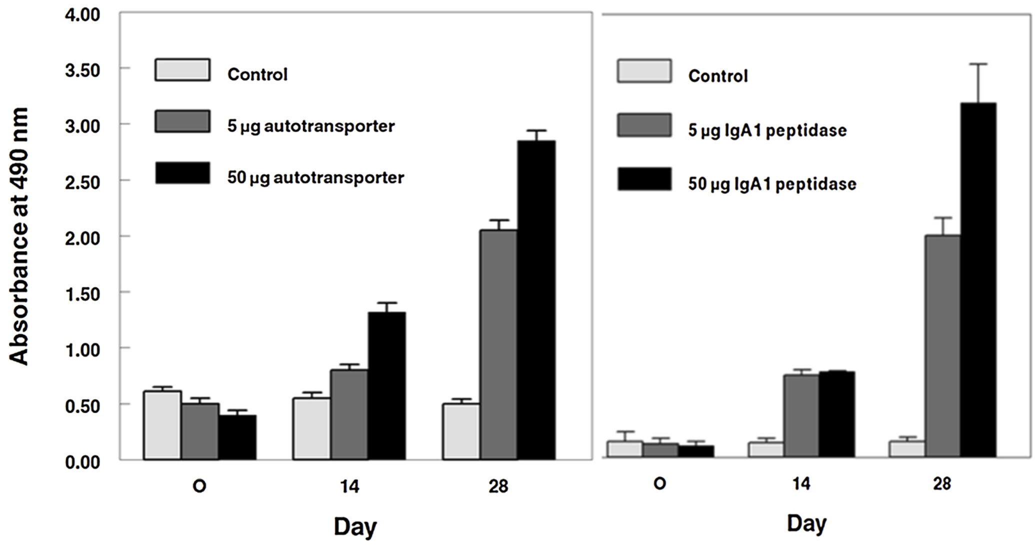

**Fig. 4.** Serum antibodies against recombinant *M. haemolytica* autotransporter and IgA1 peptidase in subcutaneously vaccinated BALB/c mice.

**Table 3**
Serum IgG anti-IgA1 and anti-IgA2 protease components (mean ng of IgG binding ± SEM) in 20 weaned beef cattle recovered from respiratory disease (Convalescent) and 20 normal, age-matched beef cattle without a history of prior respiratory disease (Control).

| Serum source | SAC 199 Autotransporter | SAC 186 PL1 Passenger AT | SAC 175 IgA1 peptidase | SAC 176 IgA2 peptidase | *M. haemolytica* Whole Cell |
|---|---|---|---|---|---|
| Control | 0.08 ± 0.04 | 0.06 ± 0.02 | 0.04 ± 0.02 | 0.05 ± 0.03 | 0.08 ± 0.03 |
| Convalescent | 0.55 ± 0.08* | 0.33 ± 0.09* | 0.28 ± 0.08* | 0.44 ± 0.10* | 2.63 ± 0.31* |

* $p < 0.01$ compared to control values.

## Declaration of Competing Interest

The authors declare that there is no conflict of interest.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vetmic.2019.108487.

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