AEM Accepts, published online ahead of print on 29 April 2011
Appl. Environ. Microbiol. doi:10.1128/AEM.02578-10

Effects of Bacterial Microflora of the Lower Digestive Tract of Free-Range Waterfowl on Influenza Virus Activation

Marcus D. King[1], M. Neal Guentzel[2], Bernard P. Arulanandam[2], Adria A. Bodour[3], Vinayak Brahmakshatriya[4], Blanca Lupiani[4,5], James P. Chambers[2§]

[1]Department of Civil and Environmental Engineering, College of Engineering, The University of Texas at San Antonio, TX 78249; [2]Department of Biology, College of Sciences, The University of Texas at San Antonio, TX 78249; [3]Air Force Center for Engineering and the Environment, Technical Division Restoration Branch (TDV), Lackland AFB, TX 78236; [4]Department of Veterinary Pathobiology, College of Veterinary Medicine and Biomedical Sciences, and [5]Department of Poultry Science, College of Agriculture and Life Sciences, Texas A&M University, College Station, TX, USA, 77843

Corresponding Author:
James P. Chambers
Department of Biology
One UTSA Circle, BSE 3.220
San Antonio, TX 78249
Phone: (210) 458-5663
FAX: (210) 458-5658
Email: james.chambers@utsa.edu

[§]Corresponding Author: james.chambers@utsa.edu

ABSTRACT

Proteolytic cleavage activation of influenza hemagglutinin (HA0) is required for cell entry via receptor mediated endocytosis. Despite numerous studies describing bacterial protease mediated influenza A viral activation in mammals, very little is known about the role of intestinal bacterial flora of birds in hemagglutinin cleavage/activation. Therefore, the cloaca of wild waterfowl was examined for *1)* representative bacterial types and *2)* their ability to cleave in 'trypsin-like' manner the precursor viral hemagglutinin molecule (HA0). Using radiolabeled HA0, bacterial secretion mediated 'trypsin-like' conversion of HA0 to HA1 and HA2 peptide products was observed to varying degrees in 42 of 44 bacterial isolates suggestive of influenza virus activation in the cloaca of wild waterfowl. However, treatment of uncleaved virus with all bacterial isolates gave rise to substantially reduced emergent virus progeny than that expected. Examination of 2 isolates exhibiting pronounced 'trypsin-like' conversion of HA0 to HA1 and HA2 peptide products and low infectivity revealed lipase activity to be present. Because influenza virus possesses a complex-lipid envelope, the presence of lipid hydrolase activity could in part account for the observed less than expected level of viable progeny. A thorough characterization of respective isolate protease HA0 hydrolysis products as well as other resident activities, i.e., lipase is ongoing such that the role of these respective contributors in virus activation/inactivation can be firmly established.

INTRODUCTION

Avian influenza viruses preferentially replicate in cells lining the intestinal tract giving rise to little or no sign of disease and high concentration of virus in the feces (9, 10, 14, 26-28, 33). 'Trypsin-like' proteolytic cleavage of hemagglutinin (HA0), a viral glycoprotein located on the surface of the surrounding viral membrane to HA1 and HA2 peptides is required for entry of the virus into the cell via receptor-mediated endocytosis (29). Although viral infection in host cells of the small intestine, colon, and cecum has been demonstrated (13, 14, 27, 33), the proteases responsible for viral activation remain unknown (11). Thus, the fundamental question arises – Could microbes present in the lower digestive tract provide proteases capable of cleaving hemagglutinin much like those found in the avian, swine, and human respiratory tracts (3, 4, 16, 17, 19, 23, 25, 30, 31)? Several studies have been carried out describing indigenous avian intestinal microflora; however, these studies focused primarily on diseases affecting commercial poultry and the potential of free-ranging birds to transport and disseminate pathogenic microorganisms to humans (5, 12, 32). Therefore, the primary focus of work described in this report assesses proteolytic cleavage of HA0 by secreted bacteria proteases in the lower digestive tract of wild ducks. Secondarily, we observed lipase activity in 2 representative bacterial secretions that could account for the inability of activated, i.e., proteolytically cleaved virus to give rise to progeny virus.

MATERIALS AND METHODS

Isolation of protease-secreting bacteria from cloacal samples. Cloacal samples were collected from 112 hunter-harvested ducks: Mallard (*Anas platyrhynchos*, *n* = 64); Blue Winged Teal (*Anas discors*, *n* = 32); Northern Pintail (Anas *acuta*, *n* = 9); and Green Winged Teal (*Anas carolinensis*, *n* = 7). Samples were collected using sterile cotton fiber swabs, suspended in 1 ml GN Broth, and transported to the laboratory. Using a 10 µl calibrated loop, samples were four-quadrant streaked onto a set of agar media selected to allow growth of a range of bacteria. MacConkey Agar (**Fisher Scientific, Pittsburgh, PA**) and Columbia CNA Agar (**Fisher Scientific, Pittsburgh, PA**) supplemented with 5 % (vol/vol) sheep blood were used to differentiate Gram-negative and Gram-positive bacteria, respectively. Detection of Gram-negative proteolytic bacteria was determined using Standard Methods Caseinate Agar (SMCA, **Fisher Scientific, Pittsburgh, PA**) (18) with modification of the published recipe by addition of 1.5 g bile salt #3 and 1.0 mg crystal violet. Gram-positive proteolytic organisms were identified using Phenylethyl Alcohol (PEA, **Fisher Scientific, Pittsburgh, PA**) Agar supplemented with 10 g sodium caseinate. Culture plates were incubated aerobically for 24-72 hrs at 37 °C and observed every 24 hrs. Colonies exhibiting differing morphologies were placed on Standard Methods Caseinate Agar and evaluated for proteolytic activity (18). Proteolytic isolates were streaked for purity on Tryptic Soy Agar (**Fisher Scientific, Pittsburgh, PA**) supplemented with 5 % (vol/vol) sheep blood.

Identification of protease-secreting bacteria from cloacal samples. Following Gram staining, isolates were identified using a Vitek 2 Compact automated identification system (bioMerieux Inc., Durham, NC). For bacterial isolates identified with confidence levels < 85 % or isolates not identified using the Vitek 2 Compact system, sequence analysis of 16S ribosomal RNA (16S

rRNA) was utilized for identification (2). Bacterial nucleic acids were isolated using a High Pure PCR template preparation kit (Roche Applied Science, Indianapolis, IN). A 1500 bp region coding for 16S rRNA was PCR amplified using conserved primers (Integrated DNA Technologies, Skokie, IL): 8F (5'-AGAGTTTGATCCTGGCTCAG) and 1492R (5'-ACGGTTACCTTGTTACGACTT). Each amplification mixture contained 24.3 µl ddH_2O, 5.0 µl 10X PCR buffer, 5.0 µl primer mix (5 µM), 4 µl $MgCl_2$ (25 mM), 4 µl premixed deoxynucleoside triphosphates (25 mM each), 2.5 µl DMSO (100 %, vol/vol), 0.2 µl Taq Polymerase (5.0 U/µl, **Fisher Scientific, Pittsburgh, PA**), and 5 µl DNA template for a total reaction volume of 50 µl. PCR cycling conditions consisted of initial denaturation at 95 °C for 5 mins followed by 30 cycles of 94 °C for 1 min, 63°C for 1 min, and 72 °C for 1.15 mins with final extension at 72 °C for 10 mins. Amplified products were purified using a High Pure PCR product purification kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions and sequenced using an Applied Biosystems 3730XL DNA Analyzer (University of Arizona). rRNA (16S) sequences were investigated using ChromasLite, and contigs constructed using ChromasPro (Technelysium Pty Ltd, www.technelysium.com.au). Sequences were compared with available GenBank sequences using the gapped BLASTN 2.2.21 program through the National Center for Biotechnology Information Server. Representative 16S rRNA sequences were submitted to GenBank under accession numbers GQ478402 to GQ478426. Identified isolates were placed in Cryocare Bacterial Preservers (Key Scientific Products, Stamford, TX) according to manufacturer's instructions and stored at -80 °C.

Preparation of bacterial supernatants containing secreted proteases. Bacterial isolates were incubated in 15 ml Brain-Heart Infusion Broth (**Fisher Scientific, Pittsburgh, PA**) for 36-72 hrs at 37 °C with shaking (250 rpm). Samples were clarified by centrifugation (9,000 x g for 10

mins) and supernatant material filtered through sterile 0.2 µm cellulose acetate membrane syringe filters. Samples were concentrated to approximately 1 ml by ultrafiltration using Centriprep 10 kDa molecular weight cut-off concentrators (Millipore, Tullagreen, Ireland). Concentrated culture supernatant material was aliquoted (100 µl) and stored at -80 °C.

Detection of protease activity in bacterial supernatants. Concentrated bacterial culture supernatants were evaluated for proteolytic activity using agar gel diffusion. Agar gels contained 25 mM Tris (pH 7.2), 150 mM NaCl, 0.6 % (wt/vol) casein sodium salt, and 1 % (wt/vol) Bacto agar poured to a depth of 4 mm (approximately 23 ml) in 100 x 15 mm Petri dishes. Aliquots (10 µl) of concentrated bacterial culture supernatant material were placed in 3 mm diameter wells and incubated for 18 hrs at 37 °C. **Plates were overlaid with 3 % (vol/vol) acetic acid and proteolytic activity noted as a clear zone or a zone of precipitated casein products (para-κ, α_{s1}-, and β-caseins) around the sample well.** Proteolytic activity was determined by measuring the diameter of the proteolytic zone around the respective sample wells. Tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK, **Sigma-Aldrich, St. Louis, MO**) trypsin (10 µg/ml) served as positive control.

Virus. A low pathogenic laboratory-derived reassortant virus construct (combination of A/Indonesia/5/2005 H5N1 and A/PR8/34 H1N1 viruses) was kindly provided by Dr. Ruben Donis from the Centers for Disease Control and was used for *in vitro* HA0 cleavage assays and *in vivo* influenza virus activation experiments. This virus contains the low pathogenic HA0 cleavage site (single basic amino acid) of of A/Indonesia/5/2005 and grows well in MDCK cell lines.

Uncleaved virus stock preparation. MDCK cells were infected with allantoic fluid-activated virus at a multiplicity of infection of 1 in **Virus Production-Serum Free Media (VP-SFM0**

(Gibco, New York). After 1 hr incubation inoculum was removed and the cells were washed 5 times with warm phosphate buffered saline (pH 7.4). Fresh **VP-SFM0** was added before incubating the cells for 24 to 48 hrs. Cell supernatants containing uncleaved virions were initially clarified by centrifugation at 8,000 rpm (Beckman Alerga 25R, A-10.250 rotor) for 20 mins at 5 °C to remove cell debris. The resulting supernatant was concentrated by centrifugation at 48,000 x g for 4 hrs at 5 °C on a sucrose cushion using a pre-cooled Beckman Type 19 rotor and centrifuge. Concentrated virus was collected and stored at -80 °C until needed.

Preparation of radiolabeled HA0. Confluent MDCK cells (**American Type Culture Collection, Manassas, VA**) were infected with allantoic fluid-activated virus at a multiplicity of infection of 1. After allowing viral attachment and penetration for 1 hr inoculum was removed and the cells were washed once with warmed PBS. The monolayers were incubated in a mixture of [^{35}S]-methionine and cysteine (0.1 mCi/ml specific activity, **Amersham Pharmacia Biotech, Pittsburgh, PA**) at 37 °C with 5 % (vol/vol) CO_2 overnight. Resulting cells and supernatant were pelleted by centrifugation at 850 x g for 2 mins and HA0 was extracted using a membrane protein extraction kit (Pierce Protein Research Products, Rockford, IL) supplemented with 100 µl of 10X protease inhibitor cocktail (**Sigma-Aldrich, St. Louis, MO**) and 1 mM PMSF **(Sigma-Aldrich, St. Louis, MO)** to prevent inadvertent cleavage of HA0 by liberated cytosolic proteases. Radiolabeled HA0 was immunoprecipitated with anti-H5 monoclonal antibodies (Rockland Immunochemicals, Inc, Gilbertsville, PA) linked to Dynabeads Protein A (Invitrogen, Oslo, Norway) per manufacturer's instructions ensuring all washes were free of protease inhibitors.

HA0 cleavage assay. [^{35}S]-HA0 (4.8 nCi, ~10,600 dpm) was treated with 10 µl concentrated bacterial supernatant and incubated for 60 mins at 37 °C. Trypsin served as positive control (10

µg/ml). Following incubation, respective samples were mixed with Laemmli sample buffer containing 5 % (vol/vol) 2-β-mercaptoethanol, boiled for 5 mins, separated by SDS-PAGE, and autoradiographed using a Typhoon 9400 Variable Mode Imager (GE Healthcare) and phosphor storage screens per manufacturer's instructions.

***In vivo* influenza virus activation.** A/Indonesia/5/2005 H5N1 reassortant virus was treated with bacterial supernatants to determine the effect on influenza virus infectivity. Uncleaved virus was diluted in PBS to a titer of 3.0 x 10^4 pfu/ml and 5 µl aliquots were incubated with 10 µl respective concentrated bacterial supernatants at 37 °C for 60 mins followed by layering on MDCK cells grown in 6-well plates for double-layer overlay analysis (34). Trypsin (2.5 µg) was used as positive control. After incubation at 37 °C for 60 mins allowing viral attachment, the inoculum was removed and the cells were rinsed with PBS. A 1.5 ml trypsin free VP-SMF0 1 % (wt/vol) agarose overlay was added to the monolayer. After 24 hrs incubation, a second 1.5 ml agarose overlay (1 %, wt/vol) containing trypsin (5 µg) was added to the wells. After incubation for 48 to72 hrs at 37 °C in a 5 % (vol/vol) CO_2 atmosphere, cells were fixed with 10 % (vol/vol) buffered formalin, the agarose layer removed, and the fixed cells stained with 2 % (wt/vol) crystal violet in 70 % (vol/vol) ethanol prior to counting. **Plaque forming unit values were determined in triplicate by the method of Gray (8).**

Assay for lipase activity. Lipase activity was determined using API ZYM substrate assay strips (bioMerieux Inc., Durham, NC). **Briefly, 65 µl of the respective supernatant preparations were added to substrate wells and enzymatic activity was determined per manufacturer's instructions.** Phospholipase C (100 µg/ml) from *Bacillus cereus* (Sigma-Aldrich, St. Louis, MO) was used as positive control.

RESULTS

Identification of protease secreting bacteria from cloacal samples: Summarized in Table 1 is proteolytic activity of the 44 bacterial isolates found in the cloaca of 67 ducks out of a total of 112 birds examined. Concentrated bacterial supernatants from *Pseudomonas aeruginosa, Pseudomonas fluorescens,* and *Bacillus subtilis* exhibited the most intense caseinolytic activity with clearing zones of 29 and 25 mm, respectively (Table **1**) while *Staphylococcus aureus* exhibited the least intense caseinolytic activity with a clearing zone of 7 mm. *Citrobacter freundii* was the only bacterial isolate that exhibited a localized clouding of the gel (no clear zone of proteolysis). Of the 67 ducks possessing protease secreting bacteria, 40 exhibited 2 or more protease secreting bacteria. Forty-five ducks were observed to exhibit no protease secreting bacteria (**data not shown**). Of the 44 bacteria identified, 11 were Gram-positive bacilli, 16 were Gram-positive cocci, and 17 were Gram-negative bacilli (Table **2**). Twenty-two of the 44 isolates were identified by 16S rRNA sequencing (Table **2**). *Aeromonas sobria* was the most frequently observed protease-secreting Gram-negative bacterium isolated from 24 of 67 samples (36 %) while *Bacillus pumilus* was the most frequently encountered Gram-positive protease-secreting bacterium (13 samples constituting 19 %, Table **2).**

HA0 cleavage by protease-secreting bacteria isolated from ducks: 'Trypsin-like' cleavage of HA0, i.e., the disappearance of HA0 with the appearance of labeled HA1 and HA2 peptides is characteristic of viral activation. Shown in Figure 1 is representative proteolytic conversion of HA0 to HA1 and HA2 peptides (approximately 58 and 26 kDa, respectively, cf. reference 24) observed for 42 of 44 isolates. 'Trypsin-like' conversion of HA0 to HA1 and HA2 peptides varied in band intensity suggesting differing degrees of hydrolysis by the respective isolates. Two of the 44 isolates appear to promote extensive

proteolysis as evidenced by complete disappearance of radiolabeled HA0 with little to no appearance of labeled HA1 and HA2 peptide bands (Fig 1, lanes 8 and 10, respectively). Although not the focus of this work, labeled hydrolysate bands migrating primarily between HA1 and HA2 peptides also differing in band intensity were also observed.

In vivo* assay:** In order to assess effects of HA0 cleavage by concentrated bacterial supernatants on infectivity of influenza virus, uncleaved virus was treated with all 44 bacterial supernatants and MDCK cell monolayers were subsequently inoculated and double-layer plaque assay analysis carried out as described by Zhirnov et al. (34). **All isolates were evaluated for toxic effects on MDCK cells. Only one isolate (*Pseudomonas aeruginosa*) was observed to exhibit deleterious effects on the monolayer (data not shown).** Shown in Fig. **2** are double-layer ***in vivo plaque assay analyses corresponding to the 10 HA0 ***in vitro*** degradation gel profiles shown in Fig. **1**. ***In vivo* data are listed from highest plaque forming unit value (left, isolate # 2) to lowest plaque forming unit value (right, isolate # 11).** The PBS control (indicated by the dotted line) corresponded to 3.4 x 10^3 pfu/ml indicating the presence of previously activated virions. Comparing the PBS and trypsin controls, the uncleaved viral stock contained approximately 12 % active, i.e., proteolytically cleaved HA0. The trypsin control (lane 1, 28,000 pfu) was in excellent agreement with the viral titer of beginning stock (30,000 pfu/ml). Surprisingly, the 10 respective bacterial supernatants shown in Fig. **2** as well as the 33 profiles not shown all gave rise to progeny pfu values less than the PBS control (***Pseudomonas aeruginosa* treated MDCK cells exhibited cytopathic effects and was not included**). In light of less than expected background progeny pfu values for endogenous activated virus following exposure to supernatants from bacterial isolates, the involvement of some additional component was suggested.

Lipase assessment. Due to the membrane enveloped nature of the influenza virus, we were desirous of examining representative isolates which exhibited low plaque forming unit values but pronounced 'trypsin-like' cleavage of HA0 for the presence of lipase activity. ***Aeromonas sobria*** **and** ***Aeromonas hydrophilia*** **isolates were assayed for lipase activity and observed to exhibit high levels (4+) of esterase (C8) and lipase (C14) activity (data not shown).** Shown in Fig. **3** (lanes 2 and 4) is the effect of these two bacterial isolate supernatants on trypsin-activated virus. Post-supernatant incubation plaque counts indicated substantially reduced infectivity when compared to the 'trypsin only' control (lane 1). Likewise, treatment of trypsin-activated virus with Phospholipase C (lane 3) indicated decreased infectivity (~ 80 %). **As shown in Fig. 4, trypsin treated, radiolabeled HA0 treated with Phospholipase C and bacterial supernatants yielded a similar cleavage pattern to that shown in Fig. 1 (lanes 11 and 12) suggesting not only competent HA0 cleavage, but no additional digestion of HA1 and HA2 peptide fragments. Although endogenous lipase activity could account in part for lower than expected emergent virus following proteolytic activation, Phospholipase C treatment and subsequent reduction of infectivity is only suggestive and not proof of lipase involvement.**

DISCUSSION

We report here that bacterial enzyme secretion mediates 'typsin-like' conversion of HA0 to HA1 and HA2 products suggestive of influenza virus activation in the cloaca of wild waterfowl. In general, bacterial supernatants produced zones of hydrolysis comparable to that of trypsin (**19 mm, Table 1**). Additionally, all bacterial supernatants were evaluated using PepTag (Promega Corp, Madison, WI) artificial peptide substrates to rule out false positives due to clouding of the agar medium arising from changes in pH (data not shown). The most frequently observed protease-secreting bacterium was *Aeromonas sobria* **(Table 2)**. In addition to *A. sobria*, three other aeromonad species identified in this study (*A. caviae*, *A. hydrophilia*, and *A. veronii*) have previously been isolated from wild waterfowl (1). Several species belonging to the genus *Bacillus* were isolated with *B. pumilus* observed in all ducks except Green-winged Teal (Table **2**). Numerous proteolytic isolates of *Enterobacter cloacae* were encountered as were isolates of *Kocuria kristinae*, formerly *Micrococcus kristinae*, and *Cellulosimicrobium*, formerly assigned to the genera *Oerskovia* and *Nocardia*.

Radiolabeled HA**0** was cleaved in 'trypsin-like' manner to varying degrees by supernatants from **42 of 44** duck cloacal isolates. **Although, 'trypsin-like' cleavage was observed, additional analysis of HA1 and HA2 peptides is required in order to rule out the possibility that small alterations arising from incorrect cleavage initially and/or subsequent removal of residues has not occurred resulting in loss of function and thus lower than expected *in vivo* infectivity data.** *Bacillus pumilus* and *Cellulosimicrobium* spp. secreted proteases that extensively degraded the HA0 glycoprotein and HA1 and HA2 peptide hydrolysis products.

Utilization of MDCK cell monolayers and the double-overlay plaque assay as described by Zhirnov and co-workers (34) simulated conditions similar to that of the lower gastrointestinal tract of birds eliminating *1)* trypsin activation of viral particles as is the case for standard plaque assays and *2)* proteases found in the allantoic fluid of embryonated chicken eggs (7); enzymes not found in distal portions of the avian intestinal tract (21). Thus, activation of virus arose solely from proteolytic cleavage by the bacterial supernatant. Interestingly, virus stock used for *in vivo* experiments contained cleaved HA0 (~12 %) which proved advantageous in that the negative effect of bacterial supernatants on these cleaved, i.e., activated virions was observed and assessed (Fig. **2**). Despite producing a 'trypsin-like' cleavage pattern, plaque counts less than that of the control which contained cleaved, i.e., activated virions was observed for all isolates tested.

Because influenza virus is surrounded by a membrane envelope, we examined the possible presence of lipolytic activity in two isolates that exhibited a **pronounced** 'trypsin-like' hydrolysis pattern but reduced infectivity of endogenous activated virus. Esterase (C8) and lipase (C14) activities were observed in both isolates. As shown in Fig. **3**, trypsin-activated virions incubated with supernatants from these two bacterial isolates (lanes 2 and 4) or Phospholipase C (lane 3) gave rise to significantly reduced plaque formation compared to the trypsin control (lane 1) albeit higher than that observed following treatment of influenza virus with supernatants from these two isolates as shown in Fig. 2, lanes 8 and 11. **Trypsin treated** radiolabeled HA**0** incubated with these bacterial isolate supernatants or Phospholipase C (Fig. **4**, lanes 3, 4 and 5) exhibited 'trypsin-like' cleavage patterns similar to those previously observed (Fig. **1**, lanes 11 and 12) **suggesting that activation, i.e., cleavage of HA0 to HA1 and HA2 peptides occurred. However, due to the complex nature of these bacterial supernatants, the**

lack of predicted infectivity could arise from contributors other than proteases either individually or in combination. For example, removal of membrane components as well as specific sugars from the glycoprotein HA0 by glycosidases present in the bacterial supernatants could also occur resulting in decreased infectivity. β-galactosidase, α-mannosidase, and N-acetyl-β-glucosaminidase activities were observed in these isolates (data not shown). Thus, disruption of the viral membrane, incorrect proteolytic cleavage, as well as possible removal of sugars required for viral binding to the cell surface receptor could account for observed disparate surveillance numbers between virus isolation and real-time PCR (6, 20, 22). Higher rates of detection are associated with molecular screening methods than that of cultured samples because PCR detects viral RNA from both viable as well as nonviable viruses (15).

Previous studies of influenza and co-infecting proteolytic bacteria in the respiratory tract demonstrated *Aerococcus viridans*, *Staphylococcus aureus*, and *Stenotrophomonas maltophilia* to activate influenza virus *in vivo* (16, 23, 30, 31). We also observed these three organisms in the avian lower digestive tract. As indicated in Figs. **1** and **2** (lane 4), *Aerococcus viridans* exhibited expected HA1 and HA2 hydrolysis products **but** with infectivity (pfu/ml) values less than that of the PBS control **like that of *Staphylococcus aureus* and *Stenotrophomonas maltophilia*** (data not shown).

In the present study, we describe identification of protease-secreting bacteria from waterfowl gastrointestinal tract and their capability to cleave HA0 both *in vitro* and *in vivo*. Despite producing 'trypsin-like' cleavage patterns consistent with that of viral activation, none of these isolates gave rise to expected progeny virus. Thus, the contribution of microbial proteases

311 to influenza activation and other bacterial derived activities, e.g., lipase to virus inactivation
312 warrants further research.

ACKNOWLEDGMENTS

Lieutenant Colonel King was the recipient of a pre-doctoral fellowship from the United States Air Force Institute of Technology. We thank Mr. John Gaines of the Air Force Institute for Occupational Health, Brooks City-Base, TX for the use of the Vitek automated identification system. Special thanks to Mr. Cory Mason and Mr. Matthew Symmank, Texas Parks and Wildlife Department, for their assistance with migratory bird collections.

REFERENCES

1. **Aguirre, A. A., T. J. Quan, R. S. Cook, and R. G. McLean.** 1992. Cloacal flora isolated from wild black-bellied whistling ducks (Dendrocygna autumnalis) in Laguna La Nacha, Mexico. Avian Dis. **36:**459-462.

2. **Bodour, A. A., K. P. Drees, and R. M. Maier.** 2003. Distribution of biosurfactant-producing bacteria in undisturbed and contaminated arid Southwestern soils. Appl. Environ. Microbiol. **69:**3280-7.

3. **Byrum, B. R., and R. D. Slemons.** 1995. Detection of proteolytic bacteria in the upper respiratory tract flora of poultry. Avian Dis. **39:**622-6.

4. **Callan, R. J., F. A. Hartmann, S. E. West, and V. S. Hinshaw.** 1997. Cleavage of influenza A virus H1 hemagglutinin by swine respiratory bacterial proteases. J. Virol. **71:**7579-85.

5. **Fallacara, D. M., C. M. Monahan, T. Y. Morishita, and R. F. Wack.** 2001. Fecal shedding and antimicrobial susceptibility of selected bacterial pathogens and a survey of intestinal parasites in free-living waterfowl. Avian Dis. **45:**128-35.

6. **Ferro, P. J., J. El-Attrache, X. Fang, S. N. Rollo, A. Jester, T. Merendino, M. J. Peterson, and B. Lupiani.** 2008. Avian influenza surveillance in hunter-harvested

waterfowl from the Gulf Coast of Texas (November 2005-January 2006). J. Wildl. Dis. **44:**434-9.

7. **Gotoh, B., T. Ogasawara, T. Toyoda, N. M. Inocencio, M. Hamaguchi, and Y. Nagai.** 1990. An endoprotease homologous to the blood clotting factor X as a determinant of viral tropism in chick embryo. EMBO J. **9:**4189-95.

8. **Gray, J.** 1999. Assays for Virus Infection, p. 81-109. *In* A. J. Cann (ed.), Virus Culture: A Practical Approach. Oxford University Press, Oxford.

9. **Hinshaw, V. S., R. G. Webster, and B. Turner.** 1980. The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. Can. J. Microbiol. **26:**622-629.

10. **Hinshaw, V. S., R. G. Webster, and B. Turner.** 1979. Water-bone transmission of influenza A viruses? Intervirology **11:**66-8.

11. **Horimoto, T., and Y. Kawaoka.** 2001. Pandemic threat posed by avian influenza A viruses. Clin. Microbiol. Rev. **14:**129-49.

12. **Hubalek, Z.** 2004. An annotated checklist of pathogenic microorganisms associated with migratory birds. J. Wildl. Dis. **40:**639-59.

353 13. **Keawcharoen, J., D. van Riel, G. van Amerongen, T. Bestebroer, W. E. Beyer, R.**
354 **van Lavieren, A. D. Osterhaus, R. A. Fouchier, and T. Kuiken.** 2008. Wild ducks as
355 long-distance vectors of highly pathogenic avian influenza virus (H5N1). Emerg. Infect.
356 Dis. **14:**600-7.

357 14. **Kida, H., R. Yanagawa, and Y. Matsuoka.** 1980. Duck influenza lacking evidence of
358 disease signs and immune response. Infect. Immun. **30:**547-53.

359 15. **Krafft, A. E., K. L. Russell, A. W. Hawksworth, S. McCall, M. Irvine, L. T. Daum,**
360 **J. L. Connoly, A. H. Reid, J. C. Gaydos, and J. K. Taubenberger.** 2005. Evaluation of
361 PCR testing of ethanol-fixed nasal swab specimens as an augmented surveillance strategy
362 for influenza virus and adenovirus identification. J. Clin. Microbiol. **43:**1768-75.

363 16. **Mancini, D. A., R. C. Alves, R. M. Mendonca, N. J. Bellei, E. Carraro, A. M.**
364 **Machado, J. R. Pinto, and J. Mancini-Filho.** 2008. Influenza virus and proteolytic
365 bacteria co-infection in respiratory tract from individuals presenting respiratory
366 manifestations. Rev. Inst. Med. Trop. Sao Paulo **50:**41-6.

367 17. **Mancini, D. A., R. M. Mendonca, A. L. Dias, R. Z. Mendonca, and J. R. Pinto.** 2005.
368 Co-infection between influenza virus and flagellated bacteria. Rev. Inst. Med. Trop. Sao
369 Paulo **47:**275-80.

18. **Martley, F. G., S. R. Jayashankar, and R. C. Lawrence.** 1970. An improved agar medium for the detection of proteolytic organisms in total bacterial counts. J. Appl. Bacteriol. **33:**363-70.

19. **McCullers, J. A.** 2006. Insights into the interaction between influenza virus and pneumococcus. Clin. Microbiol. Rev. **19:**571-82.

20. **Munster, V. J., C. Baas, P. Lexmond, T. M. Bestebroer, J. Guldemeester, W. E. Beyer, E. de Wit, M. Schutten, G. F. Rimmelzwaan, A. D. Osterhaus, and R. A. Fouchier.** 2009. Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests. J. Clin. Microbiol. **47:**666-73.

21. **Philips, S. M., and R. Fuller.** 1983. The activities of amylase and a trypsin-like protease in the gut contents of germ-free and conventional chickens. Br. Poult. Sci. **24:**115-21.

22. **Runstadler, J. A., G. M. Happ, R. D. Slemons, Z. M. Sheng, N. Gundlach, M. Petrula, D. Senne, J. Nolting, D. L. Evers, A. Modrell, H. Huson, S. Hills, T. Rothe, T. Marr, and J. K. Taubenberger.** 2007. Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. Arch. Virol. **152:**1901-10.

23. **Scheiblauer, H., M. Reinacher, M. Tashiro, and R. Rott.** 1992. Interactions between bacteria and influenza A virus in the development of influenza pneumonia. J. Infect. Dis. **166:**783-91.

24. **Skehel, J. J., and M. D. Waterfield.** 1975. Studies on the Primary Structure of the Influenza Virus Hemagglutinin. Proc Natl Acad Sci U S A **72:**93-97.

25. **Slemons, R., B. Byrum, and D. Swayne.** 2003. Bacterial Proteases and Co-Infections as Enhancers of Virulence. Avian Dis. **47:**203-208.

26. **Slemons, R. D., and B. C. Easterday.** 1977. Type-A influenza viruses in the feces of migratory waterfowl. J. Am. Vet. Med. Assoc. **171:**947-8.

27. **Slemons, R. D., and B. C. Easterday.** 1978. Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza. Avian Dis. **22:**367-77.

28. **Slemons, R. D., D. C. Johnson, J. S. Osborn, and F. Hayes.** 1974. Type-A influenza viruses isolated from wild free-flying ducks in California. Avian Dis. **18:**119-24.

29. **Steinhauer, D. A.** 1999. Role of hemagglutinin cleavage for the pathogenicity of influenza virus. Virology **258:**1-20.

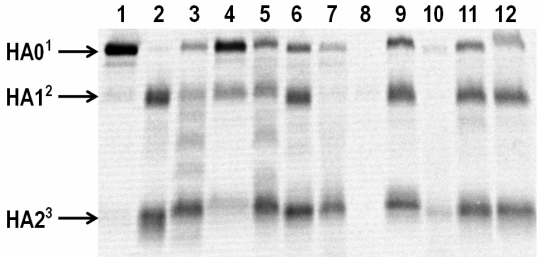
30. **Tashiro, M., P. Ciborowski, H. D. Klenk, G. Pulverer, and R. Rott.** 1987. Role of Staphylococcus protease in the development of influenza pneumonia. Nature **325:**536-7.

31. **Tashiro, M., P. Ciborowski, M. Reinacher, G. Pulverer, H. D. Klenk, and R. Rott.** 1987. Synergistic role of staphylococcal proteases in the induction of influenza virus pathogenicity. Virology **157:**421-30.

32. **Tsiodras, S., T. Kelesidis, I. Kelesidis, U. Bauchinger, and M. E. Falagas.** 2008. Human infections associated with wild birds. J. Infect. **56:**83-98.

33. **Webster, R. G., M. Yakhno, V. S. Hinshaw, W. J. Bean, and K. G. Murti.** 1978. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology **84:**268-78.

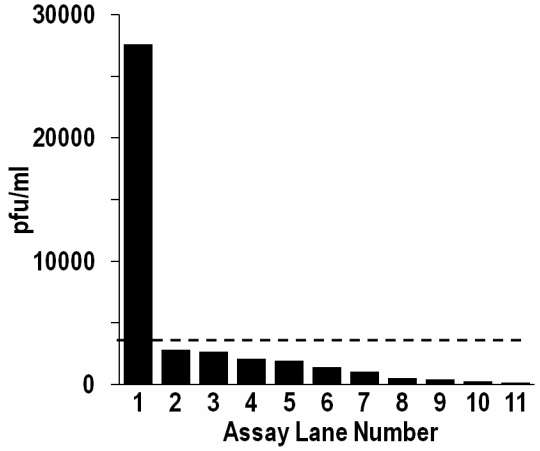
34. **Zhirnov, O. P., A. V. Ovcharenko, and A. G. Bukrinskaya.** 1982. A modified plaque assay method for accurate analysis of infectivity of influenza viruses with uncleaved hemagglutinin. Arch. Virol. **71:**177-83.



417

418

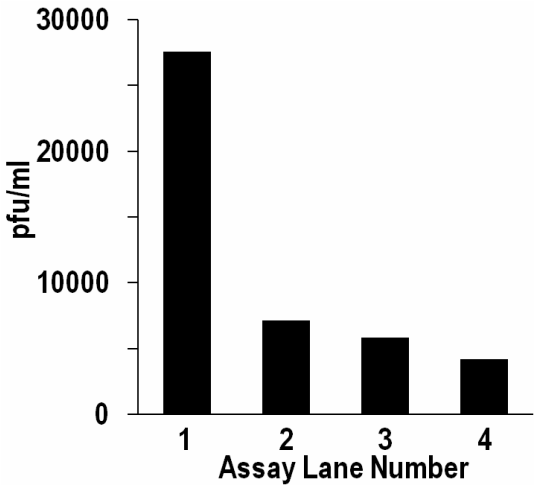
419 Figure **1**.



420

421

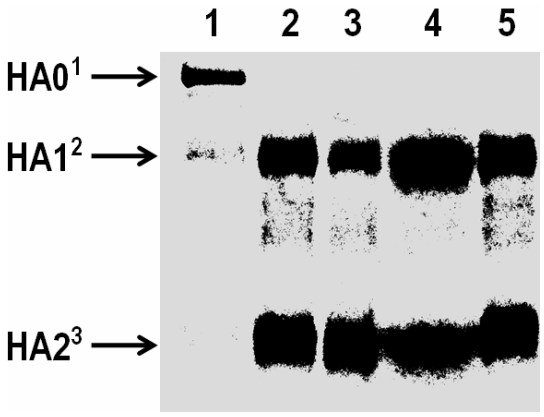
422 Figure **2**.



423

424

425 Figure **3**.



426

427

428 Figure **4**.

429 Table **1**. Proteolytic activity of bacterial supernatants.
430

Bacterial species	Diameter (mm) of clearing zone[1]
Acinetobacter haemolyticus	20
Aerococcus viridians	14
Aeromonas caviae	10
Aeromonas hydrophilia	13
Aeromonas sobria	16
Aeromonas veronii	13
Bacillus spp.	14
Bacillus amyloliquefaciens	15
Bacillus cereus	14
Bacillus coagulans	13
Bacillus licheniformis	14
Bacillus megaterium	15
Bacillus pumilus	18
Bacillus subtilis	25
Cellulomonas spp.	14
Cellulosimicrobium spp.	16
Citrobacter freundii	16[2]
Enterobacter cloacae	20
Enterococcus faecalis	20
Gemella morbillorum	16
Hafnia alvei	16
Klebsiella pneumonia pneumonia	17
Kocuria kristinae	19
Kocuria rosea	15
Lactococcus lactis lactis	23
Lysinibacillus sphaericus	11
Microbaterium oxydans	20
Microbacterium spp.	17
Pantoea agglomerans	22
Pseudomonas aeruginosa	29
Pseudomonas alcaligenes	22
Pseudomonas fluorescens	23
Raoutella ornithinolytica	20
Rhizobium radiobacter	19
Staphylococcus aureus	7
Staphylococcus cohnii cohnii	14
Staphylococcus sciuri	14
Staphylococcus warneri	14
Stenotrophomonas maltophilia	16
Streptococcus gallolyticus gallolyticus	14
Streptococcus gallolyticus pasteurianus	15
Streptococcus hyointestinalis	16
Streptococcus pneumonia	18
Vibrio vulnificus	19

431

432 Table **2**. Summary of protease-secreting bacteria identified from wild ducks.

Bacterial Species	Duck Source (n=112)[1]				
	Ducks Total	Mallard (n=64)	BWT (n=32)	Pintail (n=9)	GWT (n=7)
Gram-negative					
Acinetobacter haemolyticus	2	0	1	0	1
Aeromonas caviae	5	0	4	0	1
Aeromonas hydrophilia	13	0	13	0	0
Aeromonas sobria	24	1	18	3	2
Aeromonas veronii	8	0	7	1	0
Citrobacter freundii	2	0	2	0	0
Enterobacter cloacae	15	9	4	2	0
Hafnia alvei	6	1	5	0	0
Klebsiella pneumonia pneumonia	1	1	0	0	0
Pantoea agglomerans	4	1	1	0	2
Pseudomonas aeruginosa	4	1	0	1	2
Pseudomonas alcaligenes	2	0	1	0	1
Pseudomonas fluorescens	4	1	0	1	2
Raoultella ornithinolytica	1	0	1	0	0
Rhizobium radiobacter	2	1	0	1	0
Stenotrophomonas maltophilia	1	1	0	0	0
Vibrio vulnificus	2	0	2	0	0
Gram-positive					
Aerococcus viridians	1	0	1	0	0
Bacillus spp.	4	2(2)	1(1)	0	1(1)
Bacillus amyloliquefaciens	3	0	3	0	0
Bacillus cereus	3	3	0	0	0
Bacillus coagulans	2	0	0	0	2
Bacillus licheniformis	2	0	2(2)	0	0
Bacillus megaterium	9	1	7	0	1
Bacillus pumilus	13	3	9	1	0
Bacillus subtilis	1	1	0	0	0
Cellulomonas ssp.	1	0	1(1)	0	0
Cellulosimicrobium spp.	10	2(2)	7(7)	0	1(1)
Enterococcus faecalis	1	1	0	0	0
Gemella morbillorum	6	5	1	0	0
Kocuria kristinae	11	6	2	0	3
Kocuria rosea	3	1	0	1	1
Lactococcus lactis lactis	1	0	1	0	0
Lysinibacillus sphaericus	1	0	1(1)	0	0
Microbacterium oxydans	1	0	1	0	0
Microbacterium spp.	4	0	1(1)	1(1)	2(2)
Staphylococcus aureus	1	0	0	1	0
Staphylococcus cohnii cohnii	1	0	1	0	0
Staphylococcus sciuri	1	0	1	0	0
Staphylococcus warneri	1	0	1	0	0
Streptococcus gallolyticus gallolyticus	3	3	0	0	0
Streptococcus gallolyticus pasteurianus	1	1	0	0	0
Streptococcus hyointestinalis	2	2	0	0	0
Streptococcus pneumonia	2	1	0	0	0

433 Figure and Table Legends

434

435 Figure **1**: SDS-PAGE analysis of polypeptide fragment patterns obtained following incubation
436 of HA0 with supernatant material from protease-secreting bacterial isolates. [^{35}S]-HA0 was
437 incubated with bacterial supernatant material for 60 mins, subjected to SDS-PAGE analysis, and
438 autoradiographed as described under 'Materials and Methods'. Lane 1, PBS negative control;
439 lane 2, trypsin, 10 µg/ml; lane 3, *Streptococcus hyointestinalis* (isolate 95-11); lane 4,
440 *Aerococcus viridans* (isolate 135-8); lane 5, *Lysinibacillus sphaericus* (isolate 135-12); lane 6,
441 *Bacillus amyloliquefaciens* (isolate 135-4); lane 7, *Kocuria kristinae* (isolate 107-14); lane 8,
442 *Bacillus pumilus* (isolate 136-9); lane 9, *Enterobacter cloacae* (isolate 99-3); lane 10,
443 *Cellulosimicrobium* sp. (isolate 111-15); lane 11, *Aeromonas sobria* (isolate 124-1); lane 12,
444 *Aeromonas hydrophilia* (isolate 119-3). The numbered arrows indicate the established molecular
445 weights for HA0 and trypsin hydrolysis products HA1 and HA2 (80, 58, and 26 kDa,
446 respectively)(24).

447

448 Figure **2:** Infectivity of Influenza A/Indonesia/5/2005(H5N1) virus following incubation with
449 concentrated supernatants from duck cloacal bacterial isolates. Uncleaved Influenza
450 A/Indonesia/5/2005 (H5N1) virus was incubated with respective bacterial supernatants for 60
451 mins followed by layering onto MDCK monolayers for double-layer plaque assay analysis as
452 described under 'Materials and Methods'. 1, trypsin, 10 µg/ml; 2, *Lysinibacillus sphaericus*
453 (isolate 135-12); 3, *Streptococcus hyointestinalis* (isolate 95-11); 4, *Aerococcus viridans*
454 (isolate 135-8); 5, *Bacillus amyloliquefaciens* (isolate 135-4); 6, *Kocuria kristinae* (isolate 107-
455 14); 7, *Enterobacter cloacae* (isolate 99-3); 8, *Aeromonas hydrophilia* (isolate 119-3); 9,

456 *Bacillus pumilus* (isolate 136-9); 10, *Cellulosimicrobium* sp. (isolate 111-15); 11, *Aeromonas*
457 *sobria* (isolate 124-1); dotted line represents PBS control (3.4 x 10^3 pfu/ml).
458
459 Figure **3**: Infectivity of trypsin-activated Influenza A/Indonesia/5/2005(H5N1) virus following
460 incubation with supernatant material from two lipase-secreting cloacal bacteria isolates. Trypsin
461 treated Influenza A/Indonesia/5/2005 (H5N1) virus was incubated with bacterial supernatants for
462 60 mins followed by layering onto MDCK monolayers for double-layer plaque assay analysis as
463 described under 'Materials and Methods'. 1, trypsin only control (10 µg/ml); 2, supernatant
464 material from *Aeromonas hydrophilia* (isolate 119-3); 3, Phospholipase C only control (100
465 µg/ml); 4, supernatant material from *Aeromonas sobria* (isolate 124-1).
466
467 Figure **4**: SDS-PAGE analysis of polypeptide fragment patterns obtained following incubation
468 of trypsin-activated [^{35}S]-HA0 with supernatant material from two lipase-secreting cloacal
469 bacteria isolates. Preparation of radiolabeled HA0, HA0 cleavage with trypsin, SDS-PAGE
470 analysis, and autoradiography were carried out as previously described under 'Materials and
471 Methods'. Lane 1, [^{35}S]-HA0 untreated; lane 2, trypsin only control (10 µg/ml); lane 3,
472 Phospholipase C only control (100 µg/ml); lane 4, supernatant material from *Aeromonas sobria*
473 (isolate 124-1); lane 5, supernatant material from *Aeromonas hydrophilia* (isolate 119-3). The
474 superscripted numbered arrows indicate the established molecular weights for HA0 and trypsin
475 hydrolysis products HA1 and HA2 (80, 58, and 26 kDa, respectively)(24).
476
477 Table **1**: Detection of protease activity in bacterial supernatants was determined as described
478 under 'Materials and Methods'. [1]Values represent the diameter of the clearing zone on casein

agar produced by 10 µl concentrated bacterial supernatant material. The trypsin (10 µg/ml) control gave rise to a clearing zone of 19 mm. [2]*Citrobacter freundii* produced a cloudy zone 16 mm in diameter.

Table **2**: Identification of protease secreting bacteria from free-range ducks was achieved as described under 'Materials and Methods'. [1]BWT, Blue-winged Teal; GWT, Green-winged Teal. Numbers in superscripted parentheses indicate isolates identified by 16S RNA sequence analysis.