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ORIGINAL RESEARCH

Identification of Unique Blood and Urine Biomarkers in Influenza Virus and *Staphylococcus aureus* Co-infection: A Preliminary Study

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Abstract: Each year, there are estimated to be approximately 200,000 hospitalizations and 36,000 deaths due to influenza in the United States. Reports have indicated that most deaths are not directly due to influenza virus, but to secondary bacterial pneumonia, predominantly staphylococcal in origin. Here we identify the presence of candidate blood and urine biomarkers in mice with *Staphyococcus aureus* and influenza virus co-infection. In this pilot study, mice were grouped into four treatments: co-infected with influenza virus and *S. aureus*, singly infected with influenza virus or *S. aureus*, and a control group of uninfected mice (PBS treated). Gene expression changes were identified by DNA-microarrays from blood samples taken at day five post infection. Proteomic changes were obtained from urine samples collected at three and five days post infection using 2-D DIGE followed by protein ID by mass spectrometry. Differentially expressed genes and/or proteins were identified as candidate biomarkers for future validation in larger studies.

Keywords: biomarker, influenza virus, *Staphylococcus aureus*

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Introduction

Influenza has long been recognized as a major cause of illness worldwide and is generally characterized by fever, myalgia and respiratory symptoms.[1] While infection is usually resolved inconsequentially, current estimates indicate that influenza is responsible for approximately 36,000 deaths and 200,000 hospitalizations in the United States annually.[1–3] Recent reports indicate that most deaths are not directly due to influenza virus infection, but to secondary bacterial pneumonia,[2] predominantly staphylococcal in origin.[1,4] In children, which are a high risk group for influenza complications, co-infection with *Staphylococcus aureus* has been shown to have increased fivefold between 2004 and 2007, with older children showing higher colonization prevalence. Furthermore, co-infection with *S. aureus* in lethal childhood cases has been on the rise from 2% in 2004–2005 to 30% in 2006–2007.[5] A study found that a substantial proportion of the *S. aureus* bacteremia of infected patients was of endogenous origin, arising from colonies in the nasal mucosa.[6] The anterior nares are the major reservoir in humans of the opportunistic pathogen *S. aureus*. Approximately 20% of humans are persistently colonized intranasally, 60% are intermittent carriers, and the remaining 20% are persistent noncarriers.[7,8] Nasal colonization is asymptomatic, but is a risk for subsequent infection, especially under circumstances such as hospitalization or in immunocompromised patients.[7] Secondary bacterial pneumonia from an influenza infection has been viewed by clinicians as more difficult to treat. With the rise in co-infection, it has been suggested that influenza virus-infected patients be treated with antibiotics.[9] With the spread of MRSA and other resistant bacteria, however, antibiotics should be used in a targeted manner; only in high-risk or very ill patients with confirmed diagnosis of co-infection. To this end, identification of pertinent diagnostic or prognostic co-infection biomarkers would be invaluable. To our knowledge, there have been no published reports identifying specific diagnostic biomarkers for respiratory dysfunction due to *S. aureus*, influenza virus, or co-infection. In this preliminary study, we sought to identify the presence of candidate blood and urine biomarkers in mice with *S. aureus* and influenza virus co-infection by analysis of proteomic changes obtained from 2D differential gel electrophoresis (2D-DIGE) and gene expression changes obtained from DNA-microarrays. As these methods are commonly employed for identification of biomarkers, we believe they are appropriate tools for identifying potential markers of co-infection.

Materials and Methods

Mice

Mouse experiments were conducted using six-week old Balb/c mice from Simonsen Labratories (Gilroy, CA) and were approved by Oregon State University's (OSU) institutional animal care and use committee. In all experiments prior to intranasal infection, mice were anesthetized by intraperitoneal injection of 67 mg/kg ketamine and 4.5 mg/kg xylazine.

Virus and bacteria

Influenza A/PR/8/34 (H1N1) was obtained from ATCC and grown in MDCK cells in virus growth medium consisting of MEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 1.0 μg/ml TPCK treated Trypsin (Sigma-Aldrich, St. Louis, MO). Virus was harvested two days post-infection and stored at −80 °C for future use. Virus was titered by standard plaque assay on MDCK cells. *S. aureus* was obtained from Dr. Linda Bruslind, OSU and was grown in LB broth and titered.

Infections

Forty, six-week old, Balb/c mice were split equally into four treatment groups and infected intranasally with 50 μl of phosphate buffered saline (PBS) containing the infectious agents. Group 1 (G1) received 2×10^3 PFU Influenza A/PR/8/34 (H1N1). Group 2 (G2) was co-infected with both 2×10^3 PFU Influenza A/PR/8/34 (H1N1) followed by 1×10^6 CFU *S. aureus*. Group 3 (G3) received 1×10^6 CFU *S. aureus*. Finally, the control group, group 4 (G4) was uninfected and received only PBS. Urine was collected at three and five days post-infection and blood was collected at five days post-infection.

Proteomics

Protein profiling for urine samples collected from the four treatment groups was conducted by Applied Biomics (Hayward, CA). Samples were shipped to Applied Biomics on dry ice for 2D-DIGE. Briefly, total protein was extracted and labeled with Cy3 and Cy5 dyes and run through isoelectric focusing and sodium dodecyl sulfate–polyacrylamide gel electrophoresis

(12% polyacrylamide; 0.1% SDS) (SDS-PAGE). Two samples and an internal control were run on each gel so expression differences could be examined between gels as well as within each gel by the in-gel Decyder analysis software. Ratio change for differentially expressed protein spots between treatment groups was obtained and spots of interest were picked for identification by mass spectrometry. Protein identification was based on peptide fingerprint mass mapping (using MS data) and peptide fragmentation mapping (using MS/MS data). The MASCOT search engine was used to identify proteins from primary sequence databases.

DNA-microarray analysis

Differential gene expression from blood samples was examined by DNA-microarray analysis using a standard Affymetrix Mouse GENE 1.0ST Array (Affymetrix, Santa Clara, CA). Blood was collected at day five post infection with 50 µl of blood each from two animals collected in one Qiagen RNAprotect Animal Blood Tube (Qiagen, Valencia, CA). Total RNA was extracted using the Qiagen RNeasy Protect Animal Blood Kit. Microarray assays were performed in the Center for Genome Research and Biocomputing Core Laboratories at Oregon State University. Briefly, labeled target cDNA was prepared from 125 ng mouse blood RNA samples using the NuGen Applause WT-Amp ST RNA amplification system kit protocols (NuGEN Technologies, Inc., San Carlos, CA) and the Encore module V2. Fragmented cDNA in the amount of 2.05 µg was hybridized to the affymetrix array. Cartridge arrays protocols were followed for washing, staining and scanning of Genechips. Image processing and data extraction was performed using AGCC software version 3.0. A total of eight chips with two replicates per treatment group consisting of four pooled mice each, with the exception of only two pooled mice in a replicate of the co-infection treatment group (G2), were analyzed for expression differences using the Array Star software.

Results

Proteomics

The goal of this preliminary study was to identify potential candidate blood and urine biomarkers for future testing. From urine samples, proteomic analysis highlighted several proteins differentially regulated in the co-infection group compared to each other treatment group. A ratio of fold change in expression between groups was compared for the co-infection group against each other treatment group. The control group was compared as well to each of the groups representing a single infection. In addition, single infections were also compared. The single infection with influenza (G1) compared to uninfected control group (G4) yielded 85 spots with a fold change baseline of ≥4. The single infection of *S. aureus* (G3) compared to G4 showed 49 spots with a fold change ≥3. Co-infection group (G2) compared to G4 resulted in 106 spots ≥4 fold change while G1 and G3 compared to G2 resulted in 40 and 95 spots with ≥2.5 or 4 fold, respectively.

Twelve of the 201 spots (Fig. 1) showing the unique characteristic of having differential expression over the set baseline for the co-infection group compared to each of the other treatment groups were then picked for further analysis and identification. These criterions were established as an ideal characteristic for a protein to be a useful biomarker for co-infection, because a protein ideally would show a high expression change in co-infection compared to an individual uninfected or infected with a single pathogen of interest. Of the 12 spots identified using mass spectrometry, 11 proteins showed high confidence in the protein identification (Table 1).

Microarray analysis

Microarray analysis (Table 2) highlighted several genes of interest as potential biomarkers for validation in the future. Fold change was analyzed between the co-infection group and the other three treatment groups.

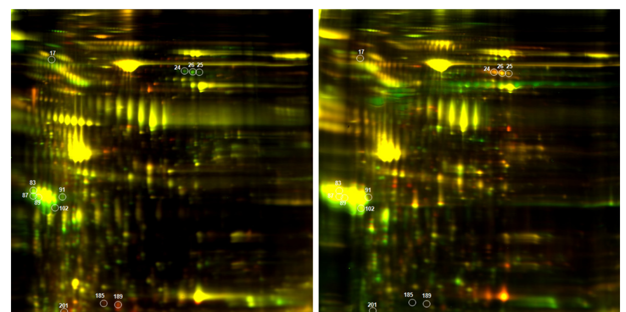

Figure 1. 2D-DIGE gels: Location of spots picked for identification by mass spectrometry on the image overlay of the two 2D-DIGE gels. Image on left is the overlay of gel images from treatment group, G1 (influenza) and G2 (co-infection). Green represents labeled proteins from G1 and red represents those labeled from G2. The image to the right is the overlay of gel images from the gel with treatment groups, G3 (*S. aureus*) and G4 (PBS). Green represent proteins from G3 and red those from G4. Imaging software was used to compare intensities, fold changes were calculated and the 12 spots highlighted above were chosen for downstream analysis.

Table 1. Proteomic data showing spot identification and predicted protein information. Protein identification with high confidence indicated by a protein score confidence interval (C.I.) of 95% or higher are indicated by an * after spot number in the first column, information such as molecular weight is the predicted data from NCBI for the proteins identified. Of the 11 proteins identified, two proteins, S100 calcium binding protein A9 (calgranulin B) and Ly-6C, were down-regulated in the co-infection group compared to each of other treatment groups. The remaining eight proteins showed up-regulation, seen by fold change differences in the co-infection group compared to the other treatment groups. The epidermal growth factor (Egf) protein and the major urinary protein 3 were both represented by distinct spots multiple times on the 2D-DIGE gels which may suggest post-translational modification of these proteins. Each treatment group is indicated by G1 (influenza infection), G2 (co-infection), G3 (*S. aureus* infection) and G4 (PBS).

Spot number	Top ranked protein name [Mus musculus]	(>4 fold) Ratio: G2/G4	(>2.5 fold) Ratio: G2/G1	(>4 fold) Ratio: G2/G3	Protein MW	Protein PI	Pep. count	Protein score	Protein score C. I. %	Total ion score	Total ion C. I. %	Accession no.
17*	Serine (or cysteine) peptidase inhibitor, clade A, member 3K	–11.3	–3.2	–10.6	46870	5.1	10	113	100	20	0	gi|16741103
24*	Egf protein	–16.7	–4.1	–8.4	131233.6	6	14	222	100	116	100	gi|17389248
25*	Egf protein	–13.7	–3.6	–8.1	131233.6	6	13	160	100	74	100	gi|17389248
26*	Egf protein	–14.9	–4	–9.8	131233.6	6	18	304	100	170	100	gi|17389248
83*	major urinary protein 3	–4.4	–3.1	–6.9	21402.7	4.7	13	447	100	246	100	gi|88196796
87*	major urinary protein 3	–5.9	–3.5	–9.8	21402.7	4.7	14	492	100	281	100	gi|88196796
89*	major urinary protein 3	–4.1	–4	–11.3	21402.7	4.7	12	648	100	449	100	gi|88196796
91*	complement factor D (adipsin), isoform CRA_d	–5.3	–2.9	–5.8	21014.2	6.1	5	286	100	227	100	gi|148699688
102*	major urinary protein 3	–10.6	–2.5	–18.5	21402.7	4.7	11	308	100	171	100	gi|88196796
185*	Ly-6C	12.9	2.7	12	14095	6.5	3	256	100	219	100	gi|387409
189*	S100 calcium binding protein A9 (calgranulin B)	8.6	3.4	16.5	13040.3	6.6	4	166	100	125	100	gi|6677837
201	ORM1-like 2	9.3	3	5.3	17378.3	9.7	2	42	0			gi|21313282

Table 2. Microarray analysis data showing expression values along with fold changes and gene identification for those genes with known annotations. Each treatment group is indicated by G1 (influenza infection), G2 (co-infection), G3 (*S. aureus* infection) and G4 (PBS).

	Gene annotation	Probe set ID	Expression level				Fold change		
			G1	G2	G3	G4	G1 vs. G2	G3 vs. G2	G4 vs. G2
≥2 fold higher in G2	hemicentin 1	10358654	6.476	7.760	6.531	6.002	2.435 ↑	2.343 ↑	3.382 ↑
	hemicentin 1	10358658	5.406	7.136	5.782	5.577	3.316 ↑	2.554 ↑	2.945 ↑
	angiopoietin 1	10428376	6.733	8.259	6.102	6.719	2.879 ↑	4.459 ↑	2.907 ↑
	zinc finger protein 442	10488459	2.755	4.109	2.870	2.872	2.555 ↑	2.359 ↑	2.357 ↑
	meiotic nuclear division 1 homolog	10499035	5.986	7.426	6.344	5.754	2.713 ↑	2.117 ↑	3.186 ↑
	immunoglobulin joining chain	10531126	6.238	7.439	6.362	6.124	2.298 ↑	2.108 ↑	2.487 ↑
	cDNA sequence BC002195	10568601	6.753	7.858	6.615	6.548	2.151 ↑	2.367 ↑	2.480 ↑
≥3 fold lower in G2	serine (or cysteine) peptidase inhibitor, clade A, member 1C	10402406	7.151	4.136	7.886	6.024	3.702 ↓	8.084 ↓	13.455 ↓
	alpha-2-HS-glycoprotein	10434689	8.554	5.964	9.559	7.749	3.444 ↓	6.020 ↓	12.077 ↓
	transthyretin	10454192	8.908	6.693	9.881	8.300	3.045 ↓	4.642 ↓	9.110 ↓
	cytochrome P450, family 2, subfamily c, polypeptide 39	10463037	6.939	4.232	8.090	5.983	3.366 ↓	6.528 ↓	14.500 ↓
	fibrinogen gamma chain	10492735	8.139	4.724	8.831	6.578	3.614 ↓	10.668 ↓	17.233 ↓
	albumin	10523062	10.353	5.685	10.688	9.367	12.840 ↓	25.427 ↓	32.079 ↓
	fatty acid binding protein1, liver	10538965	6.335	4.141	8.302	5.923	3.440 ↓	4.576 ↓	17.888 ↓
	apolipoprotein A-I	10585005	9.533	6.730	10.179	8.473	3.348 ↓	6.980 ↓	10.918 ↓
≥2 fold lower in G2	glutathione S-transferase, alpha 3	10345065	8.215	6.356	8.943	7.511	3.627 ↓	6.008 ↓	2.226 ↓
	apolipoprotein A-II	10351546	9.199	7.354	10.493	8.503	3.591 ↓	8.809 ↓	2.217 ↓
	triadin	10362422	5.389	3.031	4.104	4.864	5.127 ↓	2.103 ↓	3.561 ↓
	apolipoprotein B	10394394	7.576	5.922	9.023	6.959	3.148 ↓	8.579 ↓	2.052 ↓
	serine (or cysteine) preptidase inhibitor, clade A, member 1B-1E	10402399	7.585	5.463	8.582	6.623	4.353 ↓	8.685 ↓	2.233 ↓
	serine(or cysteine) peptidase inhibitor, clade A, member 1C	10402409	8.792	6.668	9.896	7.838	4.358 ↓	9.367 ↓	2.249 ↓
	elongation of very long chain faty acids	10408838	6.088	4.648	7.857	5.905	2.714 ↓	9.252 ↓	2.389 ↓
	carboxypeptidase B2 (plasma)	10416451	6.394	5.116	7.595	6.119	2.426 ↓	5.576 ↓	2.004 ↓
	kininogen 1	10434719	7.253	5.088	8.422	6.097	4.484 ↓	10.089 ↓	2.013 ↓
	t-complex protein 1	10441813	5.533	4.417	6.521	5.719	2.167 ↓	4.299 ↓	2.465 ↓
	expressed sequence AI182371	10482004	6.873	5.320	7.717	6.507	2.935 ↓	5.266 ↓	2.277 ↓
	carbonic anhydrase 3	10490913	8.227	6.594	10.214	7.856	3.100 ↓	12.291 ↓	2.398 ↓
	fatty acid binding protein 2, intestinal	10495820	6.606	4.608	7.705	5.662	3.994 ↓	8.551 ↓	2.075 ↓
	alcohol dehydrogenase 1 (class I)	10496438	9.150	7.440	10.064	8.472	3.272 ↓	6.164 ↓	2.045 ↓
	fibrinogen beta chain	10498981	8.060	5.229	8.462	6.708	7.114 ↓	9.401 ↓	2.787 ↓
	alpha-2-glycoprotein 1, zinc								
	cytochrome P450, family 3,	10526712	6.966	5.365	8.393	6.855	3.035 ↓	8.159 ↓	2.810 ↓
	subfamily a, polypeptide 57	10527494	5.123	3.177	6.508	4.315	3.852 ↓	10.067 ↓	2.201 ↓

(*Continued*)

Table 2. (*Continued*)

	Gene annotation	Probe set ID	Expression level				Fold change		
			G1	G2	G3	G4	G1 vs. G2	G3 vs. G2	G4 vs. G2
	group specific component	10531149	8.118	5.814	9.387	7.337	4.937 ↓	11.896 ↓	2.873 ↓
	cytochrome P450, family 3, subfamily a, polypeptide 11	10535704	5.037	3.381	6.337	4.419	3.149 ↓	7.759 ↓	2.053 ↓
	cytochrome P450, family 3, subfamily a, polypeptide 25	10535714	6.868	4.540	7.275	5.728	5.023 ↓	6.658 ↓	2.279 ↓
	pregnancy zone protein	10548207	7.869	5.716	9.317	6.801	4.445 ↓	12.134 ↓	2.121 ↓
	cytochrome P450, family 2, subfamily a, polypeptide 12	10551287	6.176	4.635	7.989	5.679	2.909 ↓	10.226 ↓	2.062 ↓
	cytochrome P450, family 2, subfamily e, polypeptide 1	10558673	8.848	5.831	9.455	6.880	8.098 ↓	12.329 ↓	2.069 ↓
	hepcidin antimicrobial peptide	10562169	8.982	5.512	9.030	6.718	11.080 ↓	11.453 ↓	2.306 ↓
	fibrinogen-like protein 1	10578352	7.822	5.328	8.136	6.601	5.633 ↓	7.002 ↓	2.416 ↓
	esterase 1	10580624	5.858	4.476	7.771	6.040	2.605 ↓	9.811 ↓	2.955 ↓
variable fold change between G2 and other treatment groups	serine (or cysteine) peptidase inhibitor, clade G, member 1	10484463	9.801	8.359	9.887	7.252	2.716 ↓	2.883 ↓	2.154 ↑
	stefin A1	10439292	11.154	9.775	8.610	7.908	2.600 ↓	2.242 ↑	3.647 ↑
	stefin A2	10439296	8.809	7.328	5.765	5.135	2.791 ↓	2.953 ↑	4.571 ↑

After removing genes with unknown annotation, eight genes of interest were identified as having expression at least 3-fold lower in the co-infection group over the three other groups, and 26 genes showed expression at least 2-fold lower in the co-infection group, not including the eight showing a 3-fold difference. A total of seven genes showed expression levels at or above a 2-fold increase in co-infection group compared to all three remaining treatment groups, and three genes showed variable expression in the treatment groups when compared to the co-infection group. Serine (or cysteine) peptidase inhibitor, clade G had expression values for the co-infection group over 2-fold higher than that of the control-uninfected group but had co-infection expression values at least 2-fold lower than that of those in either of the single infection groups. Two genes, stefin A1 and stefin A2, showed expression values for the co-infection to be at least 2-fold greater than the single infection with *S. aureus* and the control-uninfected groups, but was at least 2-fold less than the expression for the single infection with the influenza virus PR8/34 (H1N1). While there were a number of differentially expressed genes identified through the microarray, none of these genes showed a statistically significant difference by a student's t-test comparing the co-infection group to each of the other treatment groups. This is somewhat anticipated due to the level of pooling and low number of replicates.

Discussion

In this preliminary study, we have identified a number of candidate blood and urine biomarkers for the identification of a co-infection of influenza and *S. aureus*. Due to the restraints of a pilot study, we were unable to include adequate numbers of mice for sufficient statistical power. However, these results are still valuable in guiding our future work of validating the candidate biomarkers in a larger study. A serine (or cysteine) peptidase inhibitor was identified in both the proteomic and gene analysis portions of our study and may be of particular interest to look for in the future. Several of the proteins identified from urine samples showing differential regulation have demonstrated functions in viral infections/immunity. EGF protein, serine peptidase inhibitor, complement factor D, Ly-6C, and the S100 calcium binding protein A9 are all thought or have been shown to play a role in immune functions during infection.[10–15] Ly-6C, for example, is a family of murine glycoproteins that are mainly expressed by cells of hematopoietic

lineage including mature CD8+ T cells.[12] The calcium binding protein A9 has been shown in previous studies to be elevated systemically in patients with viral infections and is thought to possibly contribute to viral persistence for some RNA viruses.[13] Several genes including albumin and cytochrome P450 were found to have quite high fold changes in expression in the co-infection group compared to the other treatment groups. Following further studies, these or other genes identified in Table 2 may prove to be useful targets for a biomarker. The candidate biomarkers from this study after validation in a larger study would be potentially useful in a clinical setting to determine individuals co-infected with influenza virus and S. *aureus*. As co-infections arise more frequently, the identification of a biomarker would be invaluable for early detection of the respiratory disease and prompt treatment of co-infected individuals, and would hopefully save time, resources, and lives due to complications of an influenza and *S. aureus* co-infection. In future, it will be necessary to show statistically which if any of these possible markers may be of use as a biomarker of infection and at which stage of infection such a biomarker would be helpful in determining if a co-infection is occurring within a patient. Identification of biomarkers for MRSA co-infections would also be a useful avenue of future work that might have a beneficial purpose in a clinical setting.

Disclosure

This manuscript has been read and approved by all authors. This paper is unique and is not under consideration by any other publication and has not been published elsewhere. The authors and peer reviewers of this paper report no conflicts of interest. The authors confirm that they have permission to reproduce any copyrighted material.

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