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14. ABSTRACT

Prompt directed therapy of respiratory infections based on pathogen identification is critical for optimizing patient outcomes. This project aims to develop proactive surveillance systems which recognize disease emergence at the earliest possible stage utilizing the PLEX-ID biosensor for simultaneous detection of a wide range of pathogenic organisms. Tripler Army Medical Center and SAIC collaborated to establish this new disease surveillance technology in the Pacific Regional Medical Command. Results indicated that the PLEX-ID is able to rapidly and reliably detect influenza virus and identify substrains more efficiently than traditional diagnostics. In the second year of the program, a new broader Respiratory Virus Surveillance assay was tested to examine the ability to detect upper respiratory infections beyond influenza. Analysis of specimens collected from patients who presented with respiratory distress symptoms over a 9 month period revealed differential patterns of viruses in different seasons. In addition, specific virus types were more prevalent in certain patient groups. The PLEX-ID technology has proved to be a rapid and comprehensive detection method for viral infections allowing general disease surveillance.

15. SUBJECT TERMS

Respiratory Virus Surveillance, Pacific, PLEX-ID, biosensor

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INTRODUCTION:

Respiratory infections are the leading cause of acute illness in adults [1-2]. Prompt directed therapy based on pathogen identification is critical for optimizing patient outcome, and protecting against spread of infections in the community [3]. Unfortunately, front line clinicians currently are unable to rapidly and reliably identify infectious agents. This inability may lead to delayed treatment, improper choice of therapeutic agent, unnecessary use of antibiotics, and development of antibiotic resistance. The PLEX-ID biosensor (IBIS Biosciences, a subsidiary of Abbott Molecular Inc) enables rapid, sensitive, and simultaneous detection of a wide range of pathogenic organisms, including unexpected emerging infectious organisms [2][4-7]. IBIS-Abbott has developed a Respiratory Virus Surveillance (RVS) assay designed to provide identification of most known and emerging respiratory viruses, including, but not limited to, influenza, adenovirus and parainfluenza viruses (PIV).

The PLEX-ID assays amplify nucleic acids using primers which hybridize to conserved sequence regions of DNA which bracket unique variable sequence regions. Broad-range primers are used to amplify PCR products from different groupings of viruses. The PCR products are then detected using high-resolution electrospray time-of-flight mass spectrometry (TOF-MS) to determine the proportion of nucleotide base compositions (ratios of adenine (A), cytosine (C), guanine (G), and thymine (T)) of the PCR amplicon. The resulting "base composition signature" (BCS) of each amplicon is then matched against a database to identify the infectious agent (RT-PCR/ESI-MS; US Patent 7,217,510).

The overall goal of the collaboration between Tripler Army Medical Center (TAMC) and Science Applications International Corporation (SAIC) is to establish and deploy this new

pathogen detection and identification capability to monitor dynamic respiratory viral and bacterial propagation through Pacific Regional Medical Command populations (PRMC). In year one of this project, the PLEX-ID technology was established at TAMC. The goal for the second year of this project was to examine whether the PLEX-ID technology could be used to characterize virus patterns in patient specimens representing the general PRMC population with respiratory symptoms seen in the TAMC clinics.

BODY:

Phase 2: *Comparison of Seasonal Respiratory Virus Patterns and Dynamics in Active Duty Military and Civilians in Hawaii*

After establishing the PLEX-ID technology at TAMC, in Phase 1 of this project, in Phase 2 the primary objective was to further understand the PLEX-ID system's respiratory disease surveillance capabilities, analyzing fresh specimens collected from patients throughout a flu season. The goal of Tasks 3 and 4 of the project carried out during this second year was to be able to detect seasonal respiratory virus patterns and dynamics in a representative patient population at TAMC. Task 3 focused on establishing and verifying the ability of the PLEX-ID RVS assay to detect upper respiratory tract infections other than influenza. Task 4 concentrated on the logistics of collecting patient specimens without personal identifiers and analyzing fresh samples from patients with respiratory symptoms at TAMC.

Task 3: Verify the broad RVS assay

The outlined goals in Task 3 were completed during the timeframe covered by this report.

Changes to the Respiratory Virus Surveillance Assay for the PLEX-ID

Since the original proposal of the project, a significant amount of automation of the machine as well changes to the available assays had been made by the manufacturer, IBIS-Abbott. As such, the RVS Assay had undergone modifications. Originally the RVS plate was set to identify influenza, adenovirus, respiratory syncytial virus (RSV), bocavirus, metapneumovirus, SARS and other coronaviruses. Due to the lack of actual bocavirus specimens to verify the assay functionality, the primer pairs for this virus were removed for the latest version of the assay (version 2.5), which was used in our experiments. Instead, four new primer pairs (two sets of two) were introduced to identify human Metapneumovirus (hMPV, pp4761 and pp4762) and RSV (pp4759 and pp4760) separately, as originally these two members of the Pneumovirinae subfamily were detected together by a set of two primer pairs. This was done to increase resolution of viral detection. In addition, a third primer pair (pp5155) for Adenovirus detection was added to the plate, whose detection is in duplex with the second coronavirus primer pair. This was made possible as no interference between the two possible amplicons present was observed.

Characterization of the detection capabilities of the RVS Assay Using Patient Samples

As was done in Phase I with the PLEX-ID influenza assay, in Phase II, the functionality of the RVS assay was determined by testing 109 pre-characterized samples collected at TAMC. This included 20 adenovirus, 20 RSV, 20 PIV, 19 Herpes Simplex Virus (HSV) and 19 Enterovirus

positive as well as 11 HSV negative specimens as determined by the TAMC Department of Pathology's current gold standard testing methods. Although the RVS assay plate was designed to be able to also detect Coronavirus and hMPV, no pre-characterized patient samples were available for testing for these 2 viruses because the Department of Pathology did not check specimens for these pathogens at time of collection. The TAMC Department of Pathology standard methods entailed using a two phase methodology for respiratory virus testing using direct fluorescent antibodies (DFA). The initial testing is done directly on the patient specimen using the D3 FastPoint DFA (Diagnostic Hybrids) for Influenza A and B; PIV 1, 2, and 3; hMPV; RSV; and Adenovirus. If the specimen tests negative, it is then cultured in three cell lines: MRC5, A549, and RMK. The viral culture is subsequently tested using the D3 Ultra DFA (Diagnostic Hybrids) for the same viruses, except hMPV.

The 19 HSV positive, 11 HSV negative, and 19 Enterovirus positive samples were to serve as negative controls as the RVS plate did not have primers to assay for HSV or Enterovirus. As expected, all of these specimens (Enterovirus, HSV positive and negative virus samples) tested negative on the RVS plate. This demonstrated 100% concordance between PLEX-ID and standard lab methods in determining negative results. Further, the presence of other viruses not targeted by the RVS plate did not interfere with negative determinations and did not cause false positive results. Testing for RSV, Adenovirus and PIV with the RVS plate demonstrated that all samples were identified in concordance with the TAMC Pathology Department's standard assay results except for one Adenovirus sample which tested negative. Therefore the RVS assay sensitivity for RSV, Adenovirus, and PIV was 98.3% and specificity was 100% (see Table 1 right panel).

In addition to the 109 samples that were pre-characterized by the Department of Pathology's standard assay methods, we also tested 83 influenza negative samples from Phase 1 on the RVS plate to see if any other viruses may be detected in the influenza specimens. The rationale for doing so was that if specimens were collected based on patient presenting with respiratory symptoms, yet were found negative for influenza, there may be another pathogen causing the respiratory distress in those patients. From these 83 samples, 76 also tested negative on the RVS assay in accordance with Pathology standard assay methods results. However, 6 of the 83 influenza negative samples tested positive and other viruses were detected as follows: 1 RSV, 1 PIV, 2 hMPV, 1 Coronavirus, and 1 Adenovirus.

Table 1. Concordance of PLEX-ID Testing of Archived Samples with Pathology Standard Testing Methods.

		Pathology Viral Culture Results	
		Number positive matched out of 20 samples tested	**% Concordance**
PLEX-ID	**Respiratory Syncitial Virus (RSV)**	20/20	100%
	Parainfluenza virus (PIV)	20/20	100%
	Adenovirus	19/20	95%
	Overall	59/60	98%

Verification of Respiratory Virus Surveillance assay results with RT-PCR testing

In order to further verify the RVS assay results, 20 samples for each virus that could be detected by the PLEX-ID plate were verified by analysis using conventional RT-PCR with specific

primers for each virus. We decided to test 10 of the archived samples from Phase I plus 10 samples from the Phase II newly collected specimens that showed positive virus detection by the PLEX-ID assay. For the verification of PLEX-ID detection samples for Coronavirus, hMPV, and Influenza A & B, all 20 specimens were from the collection accumulated during Phase II (note: Coronavirus and hMPV were not always routinely tested for by Department of Pathology standard methods, so no Phase I archived samples and only samples from Phase II were used for this verification analysis). Additionally, 20 samples not containing the viruses tested for were used as negative controls. When available, Pathology results for Phase 2 samples were used for comparison between the methods. For all 7 virus species tested (140 samples), a high concordance between PLEX-ID results and RT-PCR was observed. Overall, discrepancies in results between PLEX-ID and Pathology testing indicated greater sensitivity with the PLEX-ID RT-PCR-MSI methodology.

Table 2. *Verification of RVS Assay Plate.* Characterization of agreement with specific Real Time PCR assay and standard Pathology assay results using an initial subset of positive and negative specimens indicated that the PLEX-ID agreed better with RT-PCR than Pathology results

	Plex ID positive samples (n=20 for each virus)				**Plex ID negative samples (n=20 for each virus)**			
	Real time PCR match		**Pathology match**		**Real time PCR match**		**Pathology match**	
	n	**%**	**n**	**%**	**n**	**%**	**n**	**%**
RSV	20	100%	18	90%	20	100%	20	100%
hMPV	20	100%	13	65%	20	100%	20	100%
PIV	19	95%	18	90%	19	95%	20	100%
Flu B	20	100%	17*	89%	20	100%	20	100%
Flu A	20	100%	14	70%	20	100%	20	100%
Adeno	19	95%	17	85%	20	100%	20	100%
Corona	19	95%	N/A	N/A	20	100%	N/A	N/A

*out of 19 samples due to unavailability of pathology result for one sample

Verification of RSV Assay. RT-PCR confirmed all 20 RSV PLEX-ID findings. Retrospective comparison of PLEX-ID results with Pathology results revealed that two of the samples identified by both PLEX-ID and RT-PCR to have RSV virus present, were negative by Pathology testing, indicating that standard Pathology tests missed RSV detection and that the PLEX-ID assay may be better at detecting low levels of virus that are undetectable by standard methods.

Verification of hMPV assay. For the hMPV, PLEX-ID results were confirmed by RT-PCR. Of note, seven of the 20 samples identified to contain hMPV were determined to be negative for this virus by Pathology testing, indicating that the PLEX-ID assay may be more sensitive and better at detection of hMPV.

Verification of PIV assay. For PIV testing, all of the specimens tested as positive for PIV by Pathology methods were also detected as positive by the PLEX-ID, so none of the Pathology positive detections were missed by the PLEX-ID assay. Of the two specimens that the PLEX-ID characterized as positive when deemed negative by Pathology methods, one was confirmed as positive and one determined to be negative with RT-PCR method. In addition, one specimen determined to be negative by both Pathology and PLEX-ID assays tested as positive by RT-PCR. These results indicate that standard Pathology testing method for PIV testing may be the least sensitive but most specific of the three methods. RT-PCR and PLEX-ID methods have the ability to detect PIV that may be missed by standard testing methods but both RT-PCR and PLEX-ID are not in 100% concordance with each other which may reflect the low copy threshold limits of both methods.

Verification of Influenza B assay. PLEX-ID results for Influenza B were fully confirmed by RT-PCR testing. One of the 20 Influenza B specimens did not have Pathology results available. Therefore only 19 positive samples were included in the comparison of the two methods, with 2 samples found to be negative by Pathology testing. The results for Influenza B virus thus also indicates that RT-PCR and PLEX-ID methods have the ability to detect positive samples that may be missed by standard testing methods.

Verification of Influenza A assay. All 20 PLEX-ID identified Influenza A specimens were confirmed by RT-PCR whereas standard Pathology testing methods missed detecting Influenza A in 6 of these 20 specimens. The results for Influenza A virus thus also indicates that RT-PCR and PLEX-ID methods have the ability to detect positive samples that may be missed by standard testing methods.

Verification of Adenovirus assay. Out of the 20 Adenovirus RSV assay positive samples, one was not verified by RT-PCR, possibly indicating a false positive by the PLEX-ID, as this specimen was also negative by standard Pathology testing. Two additional samples were negative in Pathology testing but found to be positive with both PLEX-ID and RT-PCR methods, again indicating that standard pathology methods is the least sensitive of the 3 methods of detection.

Verification of Coronavirus assay. For Coronavirus no Pathology results were available because this virus is not routinely tested at TAMC. With RT-PCR verification, 1 of the 20 selected Coronavirus samples tested negative.

Comparison of cycle times of the PLEX-ID versus current TAMC standard procedures for the identification of virus in patient samples

When testing upper respiratory samples with the PLEX-ID for the presence of virus, first results are available by 6 hours, representing the time span from sample preparation to detection including: 1 hour of RNA/DNA extraction, 4 hours of RT-PCR and 1 hour of processing using ESI-MS, with the end result of testing for simultaneous identification of multiple viruses. Standard laboratory testing using multiple assays, each for a single virus, yielded results in 2 to 7 days, depending on virus concentration and cytopathic effect produced to prepare a slide for fluorescent antigen testing. Thus, the PLEX-ID cycle time for detection of multiple viruses is much faster and workload efficient, enabling high throughput and rapid detection without being dependent upon cell culture limitations. Thus, the PLEX-ID is a useful tool for testing clinical specimens in disease surveillance and research. Yet, it is not a total alternative to conventional virus characterization by culture, as conventional methods enable amplification and preservation of the virus itself, allowing further and complete characterization such as whole genome sequencing.

Comparison of performance of PLEX-ID virus detection using archived versus fresh samples

The initial aim to determine whether sampling handling methods had a significant impact on detection ability of PLEX-ID assays was accomplished by simple detection of the viruses in frozen samples. Due to the logistics of the collection of fresh specimens from patients, entirely fresh nasal swabs samples could not be obtained, and thus no unfrozen specimens were run on the RVS plate. A portion of the fresh original nasal swab solution was frozen at -70°C and stored at the TAMC Department of Pathology until transfer to the TAMC Department of Clinical

Investigation (DCI) for analysis on the PLEX-ID. Depending on sample volume, storage time could vary from a couple of days to about 1 month. As was previously demonstrated with the archived specimen results reported for Phase I of this project, the RNA recovery in specimens was extremely good even after 5 years of storage at -70°C thanks to the careful handling and immediate freezing of the samples by TAMC's Department of Pathology technicians. Yet, a higher yield in copy number in fresher samples might be expected if the freezing and thawing of specimens could be avoided. Nonetheless, the logistics of obtaining specimens and storing this way until a critical mass of specimens are collected to run an assay plate cost effectively, is a realistic and more likely scenario for sample collection rather than running freshly collected samples one at a time within hours after collection.

Task 4: Surveillance of respiratory virus disease burden and determination of viral prevalence in Pacific Regional Medical Command patients using the RVS assay on the PLEX-ID platform.

Breadth of the RVS assay

To demonstrate the breath of the PLEX-ID RVS assay testing of authentic viral stocks representing each target viral group were used. As authentic viral stock is difficult to be delivered to Hawaii, we purchased the Respiratory Validation Panel 3 from ZeptoMetrix (Buffalo, NY). This panel contains all of the viruses tested in the RSV assay as well as rhinovirus and enterovirus which served as negative controls (Table 3). The viruses in this panel are all non-infectious. Unfortunately the ZeptoMetrix panel only provided for qualitative information, so no copy numbers were available. Yet, our in house RT-PCRs showed that

undiluted samples for all of the samples were at least 5 times above limit of detections for each of the viruses (see next paragraph). For each sample, the target virus served as the true positive test for sensitivity. Specificity was measured by analysis of data from viruses not present in particular samples. In this way, specificity testing was accomplished as part of the sensitivity test. The table below (Table 3) shows a summary of the ZeptoMetrix viruses tested on the RVS plate, which showed 100% sensitivity and specificity.

Table 3. *Detection of ZeptoMetrix Respiratory Virus Panel with PLEX-ID RVS Assay Plate*

Natrol Respiratory Validation Panel 3
Influenza A H1
Influenza A H3
Influenza B
Influenza A H1N1 (2009)
RSV A
RSV B
Parainfluenza 1
Parainfluenza 2
Parainfluenza 3
Adenovirus 3
Enterovirus,
Rhinovirus,
Metapneumovirus
Coronavirus 229E
Coronavirus OC43
Coronavirus SARS

	Influenza				RSV		Adenovirus			CV		hMPV		PIV 1,3		PIV 2,4	
	2798	1266	2777	1261	4759	4760	943	769	5155	2277	2562	4761	4762	2423	2425	2435	2437
FLU A H1	[33 32 25 38]	[34 22 21 24]	[33 28 18 26]	--	--	--	--	--	--	--	--	--	--	--	--	--	--
FLU A H3	[38 32 25 33]	[32 24 20 25]	[35 26 18 26]	--	--	--	--	--	--	--	--	--	--	--	--	--	--
FLU A H1N1	[39 32 24 33]	[35 21 20 25]	[34 29 16 26]	--	--	--	--	--	--	--	--	--	--	--	--	--	--
FLU B	[36 31 21 34]	--	--	[22 28 19 22]	--	--	--	--	--	--	--	--	--	--	--	--	--
RSV A	--	--	--	--	[46 24 25 40]	[21 17 10 20]	--	--	--	--	--	--	--	--	--	--	--
RSVB	--	--	--	--	[44 24 21 46]	[21 19 10 18]	--	--	--	--	--	--	--	--	--	--	--
Adenovirus	--	--	--	--	--	--	[23 32 36 21]	[33 24 37 27]	[17 27 35 18]	--	--	--	--	--	--	--	--
CV 229E	--	--	--	--	--	--	--	--	--	[38 32 23 50]	[31 32 14 29]	--	--	--	--	--	--
CV OC43	--	--	--	--	--	--	--	--	--	[40 33 18 52]	[28 30 17 31]	--	--	--	--	--	--
CV SARS	--	--	--	--	--	--	--	--	--	[36 35 23 49]	[33 27 17 29]	--	--	--	--	--	--
hMPV	--	--	--	--	--	--	--	--	--	--	--	[43 24 20 33]	[41 20 14 26]	--	--	--	--
PIV 1	--	--	--	--	--	--	--	--	--	--	--	--	--	[58 44 20 31]	[24 19 15 19]	--	--
PIV 2	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	[39 22 13 25]	[40 30 31 45]
PIV 3	--	--	--	--	--	--	--	--	--	--	--	--	--	[66 33 20 34]	[23 22 14 18]	--	--
Rhinovirus	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--
Enterovirus	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--

Limit of detection (LOD) for the RVS assay virus detection

To understand the detection capabilities of the RVS assay, the LOD needed to be established. Earlier in 2011 the Department of Infectious Disease at the John Hopkins Institute had published a paper [8] that investigated the LOD for the PLEX-ID RSV assay version 2.0. This version still contained the primer pairs for the Bocavirus and the different primer sets for the differentiation of hMPV and RSV. From this paper we adapted the limit of detection for Influenza A and B, Parainfluenza 3, 2 and 4 as well as SARS as the primer pair combination had not changed in the

newer version of the assay. For the following LOD experiments, reference viruses from ZeptoMetrix were used, and actual copy numbers were determined by RT-PCR (PrimerDesign, South Hampton, UK). In accordance with the methods of Chen et al , we determined the limit of detection for hMPV (15 copies) and Adenovirus (7 copies). For hMPV, a total new set of primer pairs and for Adenovirus an additional primer pair was used in the assay 2.5 version (Table 4). So, for RSV we were not able to determine our own LOD as no reference virus with high enough titer was available. In consultation with IBIS-Abbott, the internal control LOD of 15 copies was used as a cut off in our studies. Even though the primerpairs for PIV 1 and 3 are the same, we also titrated the LOD for PIV 1 (15 copies) as this virus serotype was not covered in the Chen paper. For the Coronavirus, only SARS had been tested in the publication, so we additionally tested Coronavirus Group 2 and 1. Similar to SARS, limit of detection for Coronavirus Group 2 was 150 copies. Of note, the copy numbers for Coronavirus Group 1 were always significantly lower in the PLEX-ID measurement when compared to RT-PCR testing, indicating the affinity of the Coronavirus primer pairs might be diminished for Coronavirus 1 serotype in comparison to Coronavirus Group 2 or SARS. As only a couple of the Coronavirus samples were CoV1, we decided to verify all of these samples by RT-PCR testing and not to determine a LOD. Once the different limits of detection were assessed, they were set as assay cut off points. If copy numbers of a sample were below this level, the sample was considered to be negative.

Table 4. *Limits of Detection for RVS assay*

Respiratory Virus	LOD	Source
Influenza A	60	Chen et al. 2011
Influenza B	70	Chen et al. 2011
Respiratory Syncytial Virus	15	Abbott consult
Adenovirus	7	Experimentally determined
Coronavirus Group 1	Confirm by RT-PCR	Experimentally determined

Coronavirus Group 2	150	Experimentally determined
Coronavirus SARS	125	Chen et al. 2011
Human Metapneumovirus	15	Experimentally determined
Parainfluenza 1	15	Experimentally determined
Parainfluenza 2,4	150	Chen et al. 2011
Parainfluenza 3	70	Chen et al. 2011

Analysis of virus patterns in routine samples from TAMC patients with respiratory symptoms

During the months of October 2010 through June 2011, the Pathology Department at TAMC collected 864 specimens from patients with symptoms of upper respiratory infections. These samples were cultivated for the appropriate standard pathology fluorescence antibody testing. A part of the original nasal swab was frozen at -70°C before transport to DCI for sample analysis on the PLEX-ID RVS assay. RNA/DNA was extracted, followed by RT-PCR amplification and PLEX-ID testing. For all of the 864 samples, results were collected and stored in a SQL database developed as part of this program, (see later paragraph) for use in further analysis. De-identified patient data was obtained from the CHCS system at TAMC including Pathology results, duty status, date of collection, age of patient and branch of service. This data was merged with the PLEX-ID findings into the SQL database.

Patient data for 848 specimens has been collected thus far. The data for the remaining 16 samples will be incorporated in the dataset once available and are not used in the demographic analysis part of this report. 330 (39%) out of the 848 samples were positive for virus detection. From the 330 samples, 24 (7.3%) showed detection of more than one virus type.

Analysis of general viral prevalence, by age, patient category and time-span

Out of the 848 samples included in this analysis, detection of RSV was most common (13%, 113 samples), followed by Influenza A (8%, 70 samples) (Figure 1).

Figure 1

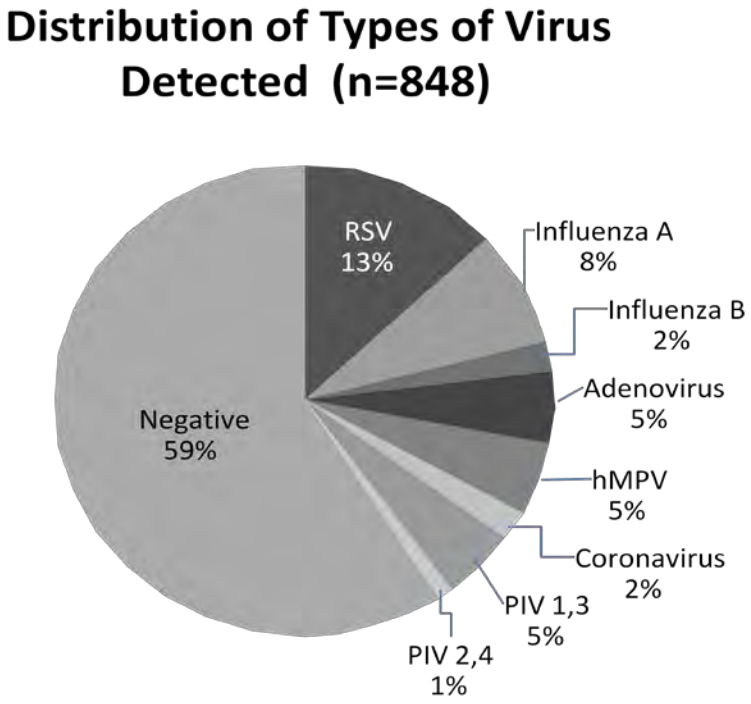
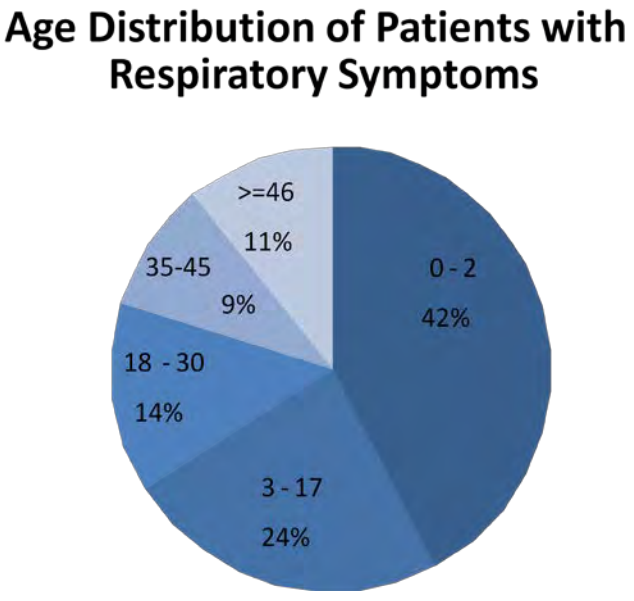


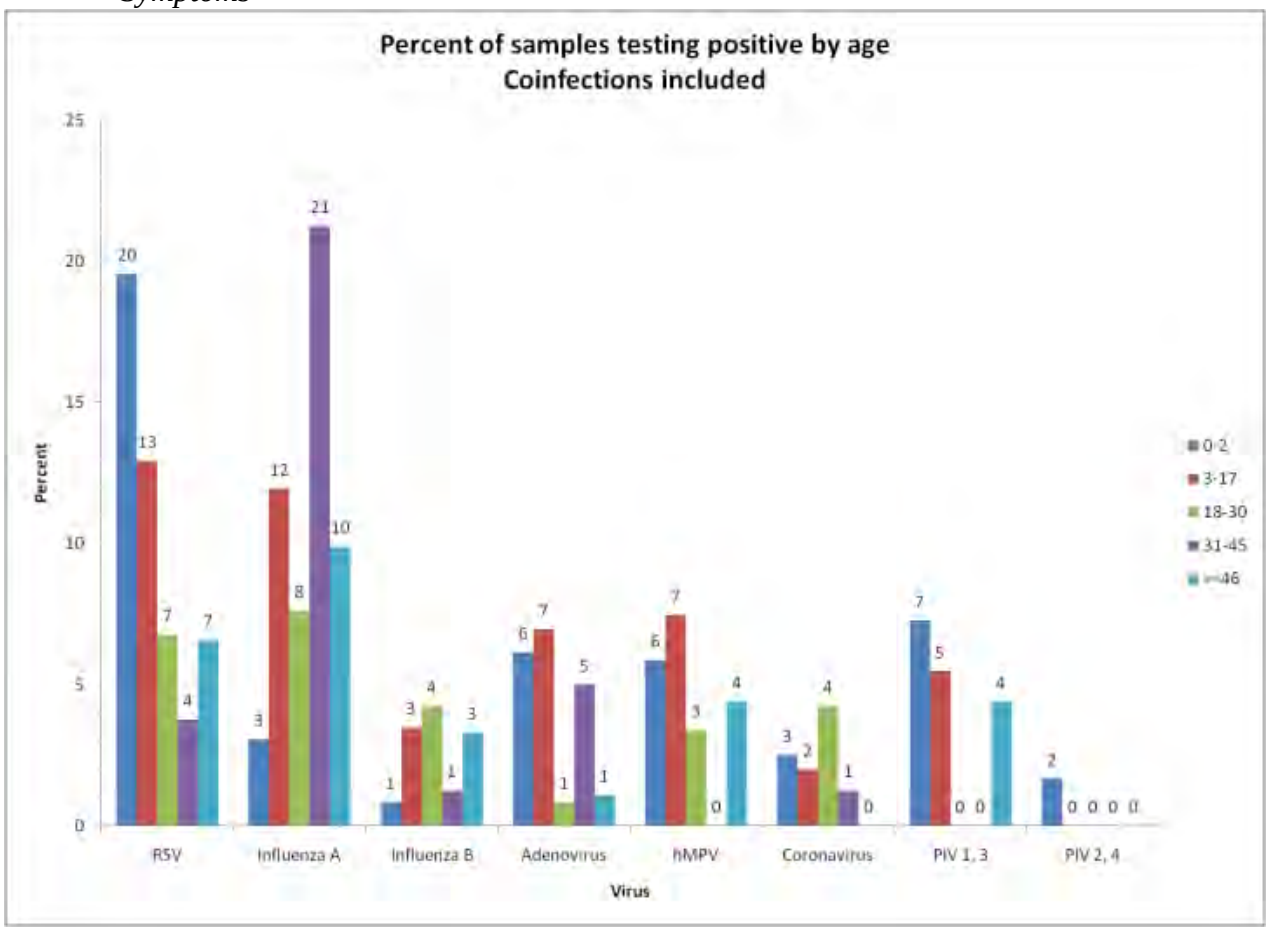
Figure 2



Most samples were collected from the 0-2 years of age group (43 %, 358 samples) followed by patients 3-17 years old (24%, 201). Within the age groups, percentage of positive samples varied significantly from 47% positives in the 2 to 17 year old patient group to 27% in the 18 to 30 year old patients.

Distribution of the different virus species showed interesting patterns concerning the prevalence in different age groups. RSV was most common in children under the age of 2, and it is the most common cause of bronchiolitis and pneumonia in children under 1 year of age in the United States (http://www.cdc.gov/rsv/). Of note, Influenza A was mostly seen in patients aged 31 to 45 years. From our findings, PIV 1 and 3 seems to be more prevalent in young people (0-17) or in older patients over the age of 47 years. PIV is the known cause for croup (PIV1/2) and pneumonia in small children and infants and is the cause for repeated infection of the lower respiratory tract especially in older adults (http://www.cdc.gov/ncidod/dvrd/revb/respiratory/hpivfeat.htm). For the other viruses, no clear trends were observed due to the small numbers of positive samples per virus per age group.

Figure 3. Patient Age Distribution within *Virus Type Detected in Patients with Respiratory Symptoms*



Patient Sample Distribution

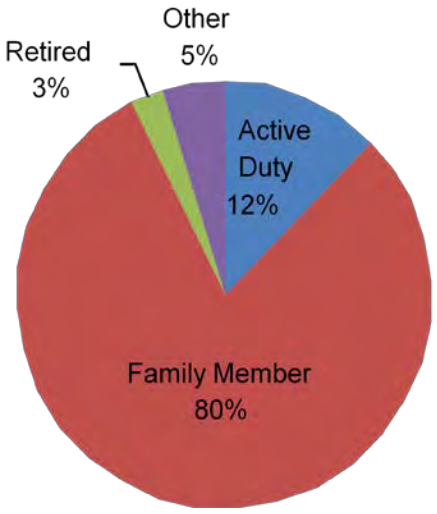


Figure 4.

To design future epidemiological surveillance studies, we needed to understand the distribution of the population of patients with respiratory symptoms seen at TAMC primary care clinics as represented by the specimens collected in this study. The dataset was thus analyzed by patient category. We found that the vast majority of the samples collected were from family members, including children and spouses of active and retired military and personnel (681 samples, 80%). The number of positive samples is highest in this category, as it contains all of the patients age 0-17 which were identified to have most positive virus results.

Active duty soldiers

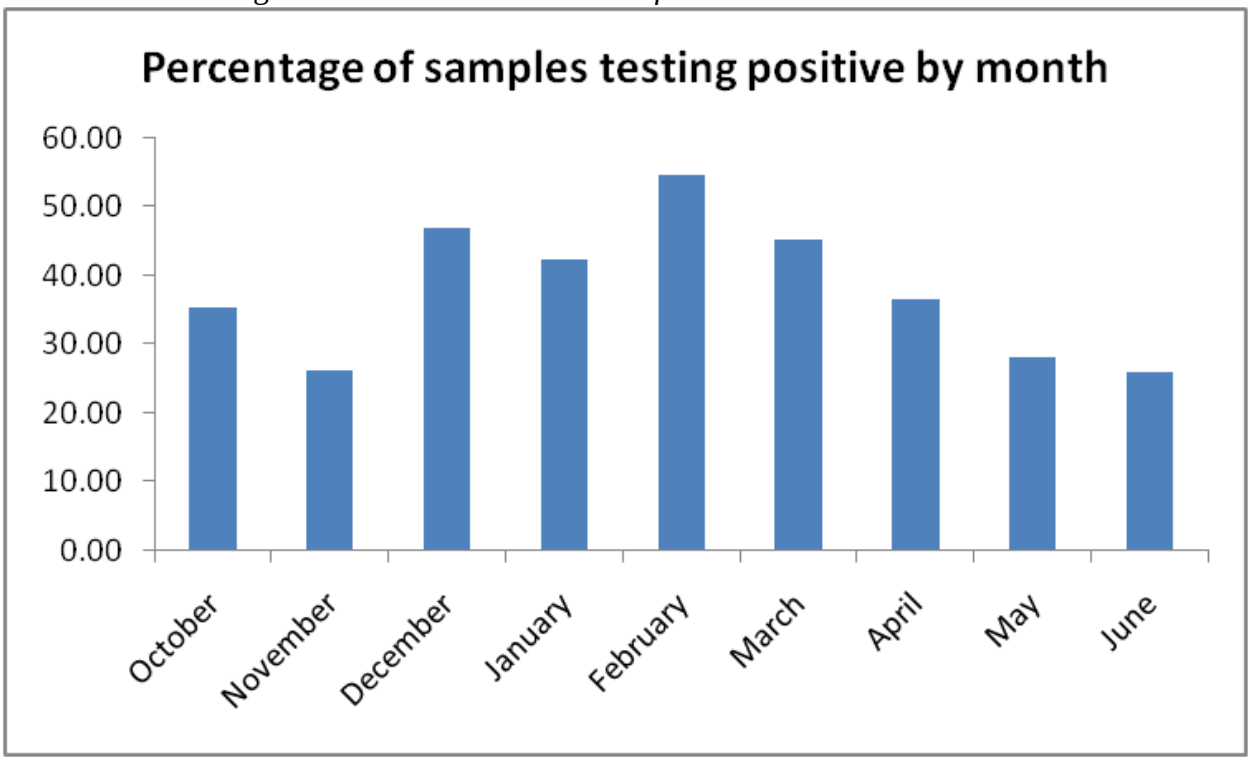
From the 848 samples collected, 105 (8%) specimens were from active duty soldiers. This gives us an indication of the representative catchment of patients for epidemiological surveillance of active duty military if only specimens from patients presenting at TAMC primary care clinics are obtained. Out of the 105 specimens, 21 swabs tested positive for a respiratory virus. Most common was Influenza A (8%) followed by Coronavirus (5%) (Table 5). As the number of positive samples is small, no further sub-analysis was conducted.

Table 5. Virus types of 21 positive specimens collected from Active Duty Military Personnel

Virus	n	% of positive specimens
Influenza A	4	19.0%
Influenza B	8	38.1%
Adenovirus	0	0
hMPV	1	4.8%
Coronovirus	2	9.5%
PIV 1,3	5	23.8%
PIV 2,4	0	0

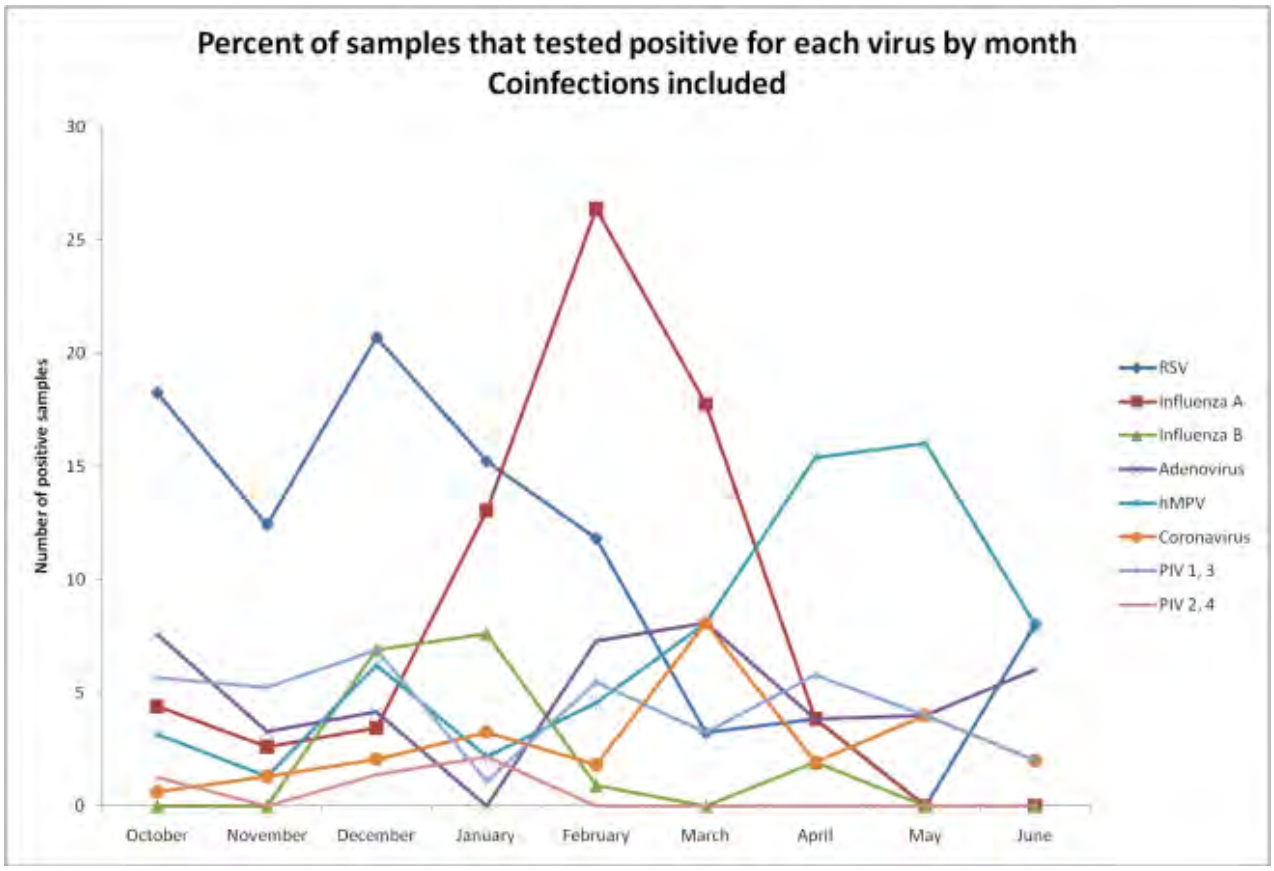
One of the goals of setting up a Respiratory Surveillance Program for the Pacific Region is to determine whether seasonal differences in virus infections match the patterns observed in the rest of the United States. This information is of pivotal importance when making decisions on type and timing of vaccinations. We thus examined the virus profile of specimens collected by month. Most patients presented respiratory symptoms in the TAMC primary care clinics and thus specimens were collected, during the months of October through February (Table 12), with the percentage of most positive samples detected in February (55%) (Figure 5).

Figure 5. *Distribution Patterns of Virus Detection Over Time*



We assessed the distribution of the different virus detections over time. The percentage of samples positive for each virus per month can be seen in Figure 6.

Figure 6



At the beginning of the collection time span, RSV showed high detection rate in October through December 2010 (19 to 30 positive samples per month), while most other viruses barely reached double digits in detection. After December, a sharp decline in RSV infections can be observed tapering down to 2 to 4 detections per month ($p<0.05$). Interestingly, the RSV infection pattern reported by the CDC for this region differs in regards to lower RSV infection reported in October, with a steady increase during November and December peaking through the months of January and February with a decline in infections in the following months (http://www.cdc.gov/surveillance/nrevss/rsv/state.html). Of note, the Pacific Region described on the CDC site included the states California, Washington, Oregon, Alaska and Hawaii, with only three reporting sites out of 60 from the state of Hawaii. This suggests that Hawaii does not

follow the pattern for RSV infection of the Pacific Coast states, but rather has an earlier onset (before October), peak and decline.

Influenza B detection started picking up in November, peaking in December to decline afterwards until March followed by sporadic detections throughout the rest of the time period ($p<0.05$). Throughout early winter, Influenza A was detected in a low frequency, while infections with Influenza A picked up in December through January peaking in February with being the virus with the highest incidents. After February, Influenza A detection declines until it is not detected anymore in May and June ($p<0.05$). Of note, Influenza A closely follows the pattern for flu infection observed in the continental United States (http://www.cdc.gov/flu/weekly/). Yet, Influenza B detection at TAMC is off-set to the continental US (and to TAMC Influenza A), peaking in December and basically disappears by the time influenza A is peaking in February. Yet, the small overall sample size for Influenza B detection ($n=19$) might be partially responsible for the differences observed.

For most months, hMPV detections did not reach more than five samples, except in December and May where samples tested positive for hMPV. Interestingly, during April and May, hMPV was the predominate virus to be seen ($p<0.05$). This is most likely due to the reduced amount of overall samples tested as well as less positive detections within these specimens.

For all of the other viruses (Adenovirus, Coronavirus and PIV) no significant association during the time span measured was observed, indicating that these viruses might not have a seasonal pattern and cause upper respiratory infections all year round in continuous manner.

Analysis of virus detection with the PLEX-ID revealed interesting aspects concerning the seasonal detection of the different virus types known to be responsible to cause upper respiratory infection. It will be of interest, comparing the findings at TAMC to infection patterns observed in the State of Hawaii overall or other DOD surveillance areas as this information was not available at time of reporting.

Co-infections

One key advantage of the PLEX-ID over conventional virus detection methods is the capability to more easily detect multiple viruses in the same sample. From the 330 positive samples, analysis identified 24 samples (7.3%) with primer pair amplification for more than one virus. Of these 24 samples, 19 (79%) contained RSV in combination with various other viruses (Table 6). The remaining 5 sample combinations are also listed below.

Table 6. Percentage of samples with possible co-infections

Infection 1	Infection 2	# of samples	%
RSV	PIV 3	4	16.67
RSV	hMPV	6	25.00
RSV	Influenza A	4	16.67
RSV	Adenovirus	3	12.50
RSV	Coronavirus	2	8.33
Adeno	PIV3	2	8.33
hMPV	Adenovirus	1	4.17
hMPV	Coronavirus	1	4.17
hMPV	PIV3	1	4.17

The primer pair compositions in these 24 samples are heterogeneous throughout all of the mixed viral detections and no particular trends in specific genotypes concerning any of the viruses can be observed. Additionally, 21 of the 24 samples (87.5%) are in children aged 0-17, leaving the rate of co-infection in this age group at 8.5%. Of note, over 70% of the samples from children with co-infections were collected at TAMC's Emergency Room, indicating that these pediatric

patients might have suffered from more severe disease symptoms. Several studies have addressed this issue of co-infections in children, and data show that these are fairly common especially in children aged 1 to 5. Detection of two viruses in specimens collected in a daycare setting as well as children admitted to an intensive care unit showed that co-infection rates can be as high as 27 and 35%[9-10]. Similar to our data, especially RSV seems to occur in conjunction with other common infection causing agents including rhinovirus. This virus is not included in the RVS assay at the moment, and might account, at least partially, for the higher rate of co-infections observed in this paper.

The data suggest that co-infections with several viruses are common in pediatric patients, and it would be of interest to additionally test those samples for the presence of bacterial species also known to cause upper respiratory infections. This is of interest, as over 60% of the pediatric specimens are collected in an Emergency Room setting, suggesting an acute and severe disease pattern in these children.

Comparison of PLEX-ID results with Pathology testing

Assessment of concordance between Pathology test results and PLEX-ID findings was conducted on 824 samples, excluding the 24 samples that had co-infections to reduce bias between two methods.

Additionally, Coronavirus is not listed as Pathology does not test routinely for this virus. Table 7 summarizes the sensitivity (% of pathology positives) and specificity (% of pathology negatives) when setting the Pathology results as gold standard. Specificity for all of the viral species ranks between 94.2% for RSV and 99.8% in Influenza B. Sensitivity ranged from 74.4% for PIV detection to 92.5% for RSV. Interestingly, even though numbers of PIV positive samples are

similar for PLEX-ID and Pathology testing, only three quarters of each were positive in both methods accounting for the low sensitivity. On the other hand, almost twice as many samples were identified as RSV positive with the PLEX-ID, almost all of the RSV samples tested positive by Pathology were positive my PLEX-ID. Hence, the specificity was the lowest among all viruses tested.

Table 7. Comparison of findings between TAMC Pathology and PLEX-ID results

	Plex ID		Pathology		Positive on both plex and pathology		Negative on both plex and pathology	
Coinfections removed (n=824)	n	% positive	n	% positive	n	% of pathology positives	n	% of pathology negatives
RSV	94	11.4	53	6.4	49	92.5	726	94.2
Influenza type A	66	8.0	64	7.8	58	90.6	752	98.9
Influenza type B	19	2.3	19	2.3	17	89.5	803	99.8
Adenovirus	36	4.4	30	3.6	27	90.0	785	98.9
hMPV	35	4.2	25	3.0	23	92.0	787	98.5
PIV	40	4.9	39	4.7	29	74.4	774	98.6

Any discrepancies between the PLEX-ID and Pathology findings were further investigated using real-time RT-PCR assays as a third method for result confirmation. From the 824 samples with available Pathology results, 108 specimens showed differences in virus detection. From these 108 samples, 91 specimens were tested by RT-PCR to date; remaining RT-PCR confirmations are still underway. (Table 8).

Table 8. Comparison of findings between TAMC Pathology and RT-PCR results

	According to Pathology		**According to RTPCR**	
	False Neg	**False Pos**	**False Neg**	**False Pos**
Adenovirus	1	8	0	7
Influenza A	1	8	0	0
Influenza B	2	2	1	0
hMPV	1	9	0	2
PIV	5	11	1	8
RSV	4	43	0	29
sum	14*	81*	2	46

*Sum is 95 (4 samples were either false positive or false negative)

From the discrepant Adenovirus samples, the false negative samples were confirmed negative by RT-PCR, yet only 1 out of the 8 false positive samples also tested positive. Of note, 5 of 8 PLEX-ID false positive Adenoviruses had copy numbers close to the detection limit (7-15 copies).

RT-PCR results for the Influenza A samples confirmed all of the PLEX-ID findings. For Influenza B only three of the 4 PLEX-ID findings were proven true. Yet, the second false negative sample did show a detection by the PLEX-ID, but was deemed negative due to detection below limit of detection (59.5545 copies, LOD is 60 copies).

For hMPV, the false negative PLEX-ID sample also tested negative in the RT-PCR, and RT-PCR also confirmed the PLEX-ID finding for 7 of 9 false positive samples. Of note, the two samples that tested negative by RT-PCR had low level copy numbers by PLEX-ID, (23 and 50 copies), whereas the other seven specimens had PLEX-ID copy numbers ranging from 341- 1764 copies.

PLEX-ID results for the false negative PIV samples were confirmed in 4 of 5 cases. Only 3 of 11 false positive samples also tested positive by RT-PCR result. Of note, the 8 false positive PLEX-ID samples only one of 2 primer pairs showed amplicon detection.

From all the viruses tested, RSV had the highest number of discrepant samples, with 4 false negative and 43 false positive specimens. For the 4 false positive samples, PLEX-ID results were confirmed by RT-PCR. Of the 43 samples identified as negative by Pathology but positive by PLEX-ID, 14 confirmed the PLEX-ID finding. Of the 29 samples still falling into the category of false positives, 15 of 29 did have RT-PCR detections; however, these detections fell below the RT-PCR cutoff value of 10 copies. The other 14 of 29 had no RT-PCR detections. Of note, limit

of detection for RSV was established by testing of the synthetic construct IBIS uses for copy number determination in the RVS assay and might not reflect the "true" LOD with biological samples. Yet, no RVS virus stock with high enough copy numbers (e.g. ZeptoMetrix) is available at this time.

When comparing overall sensitivity and specificity (Table 9), results indicate that the PLEX-ID is the more sensitive method of detection, as more true positive samples are identified. However, with the higher sensitivity, a higher frequency of false positive samples is observed with the PLEX-ID reflected by the lower positive predictive values, mainly for adenovirus, PIV and RSV. These numbers can be improved especially in the case of RSV by more extensive testing concerning the true limit of detection with biological samples.

Table 9. Comparison of PLEX-ID and Pathology Assays for Virus Detection against RT-PCR

		Real-time RTPCR			
		Sensitivity	Specificity	PPV	NPV
PlexID	Adenovirus	100	99.1	80	100
	Influenza A	100	100	100	100
	Influenza B	95	100	100	99.87
	hMPV	100	99.74	93.75	100
	PIV	97.06	98.97	80.49	99.87
	RSV	100	96.11	68.48	100

		Real-time RTPCR			
		Sensitivity	Specificity	PPV	NPV
Pathology	Adenovirus	96.43	99.87	96.43	99.87
	Influenza A	87.88	99.87	98.31	98.93
	Influenza B	90	99.87	94.74	99.75
	hMPV	76.67	99.87	95.83	99.11
	PIV	91.18	99.48	88.57	99.61
	RSV	77.78	99.46	92.45	98.15

Influenza virus analysis

89 of the 848 samples tested positive for influenza with 70 Influenza A and 19 Influenza B. Identification for influenza strains (H/N classification) is well in the capabilities of the PLEX-ID given by the combination of the different amplicon compositions for the different primer pairs. Yet, determination of the strain genotype (single entry in the Genbank) is not unbiased, as several genotypes can have the same amplicon composition combinations. Yet, vaccination

recommendations are given for specific genotypes and those genotypes are included in the vaccine for the up-coming flu season. The flu vaccine for the 2010/2011 contained following strains:

- Influenza A California/7/2009 (H1N1) (pandemic)
- Influenza A Perth/16/2009 (H3N2)
- Influenza B Brisbane/60/2008

Unfortunately only Influenza A California/7/2009 (H1N1) pandemic is the only strain included in the database for the PLEX-ID, as the whole genome was available in Genbank. For neither the A/Perth nor the B/Brisbane strains are in the PLEX-ID database because only segments 4 (HA) and 6 (NA) are available from Genbank. The primer pairs used for identification of Influenza A and B do not bind in these regions, so no alignment was possible. Therefore, we were not able to analyze the Influenza A H3N2 and Influenza B vaccination efficiency as no primer pair compositions were available for comparison. Yet, the different base pair compositions observed for these two influenza subtypes are listed in Tables 10 and 11.

Table 10. Base pair compositions for Influenza B samples

suggested genotype	other genotypes	1261	2798
Influenza B virus B/BARCELONA/215/2003	7	[26 23 19 23]	[36 31 21 34]
Influenza B virus B/Hawaii/11/2004	14	[26 23 19 23]	[36 31 20 35]
Influenza B virus B/Taiwan/539/2005	10	[22 27 20 22]	[37 31 20 34]

Table 11. Base pair compositions for Influenza A samples

suggested genotype	other genotypes	1266	2777	2798
Influenza A virus A/Thailand/CU-H16/2009(H3N2)	none	[31 24 21 25]	[34 29 16 26]	[40 31 24 33]
Influenza A virus A/Thailand/CU-B1697/2009(H3N2).	none	[31 24 21 25]	[36 26 17 26]	[41 30 24 33]

For the 19 Influenza B samples, only three different amplicon compositions were observed, with first recognition for the three compositions were between years 2003- 2005,

19 of the 70 Influenza A were of the H3N2 subtype. All 19 samples had the same amplicon composition combination for primer pairs 1266 and 2777 and only one sample differed for 2798 (Table 11). These primer pair compositions are very unique in the sense that no other entries in Genbank have the same combinations of primer pair compositions, indicating that are very specific substrain propagated during the 2010/2011flu season within the TAMC patient population. Yet, it cannot be excluded that these specific genotypes observed might have been the Influenza A/Perth substrain.

The remaining 51 samples contained all Influenza A H1N1-pandemic. Within this group only 4 distinct amplicon compositions for the three primer pairs were detected (Table 12).

Table 12. Distinct amplicon compositions for Influenza A H1N1 samples

suggested genotype	other genotypes	1266	2777	2798
Influenza A virus A/MANAGUA/0536N/2009(Pandemic-H1N1)	29	[35 21 20 25]	[34 29 16 26]	[39 32 25 32]
Influenza A virus A/CALIFORNIA/04/2009(Pandemic-H1N1)	20	[35 21 20 25]	[34 29 16 26]	[39 32 24 33]
Influenza A virus A/HIROSHIMA/201/2009(Pandemic-H1N1)-PB1-S2 SNP	1	[35 21 20 25]	[34 29 16 26]	[38 33 24 33]
Influenza A virus A/ALABAMA/02/2009(Pandemic-H1N1)-PB1-S1 SNP	1	[35 21 20 25]	[34 29 16 26]	[40 31 24 33]
Influenza A virus A/CALIFORNIA/07/2009(Pandemic-H1N1)		**[35 21 20 25]**	**[34 29 16 26]**	**[39 32 24 33]**

Similar as with Influenza H3N2, primer pairs compositions for 1266 and 2777 are the same in all samples, only 2798 (binding in PB1 segment) shows some variations by Adenine to Guanine and Cytosine to Thymidine single nucleotide polymorphisms. Of note, 20 of the H1N1 Influenza A samples show the exact genotype than the Influenza A California/07/2009 strain used for vaccination. Similarly, the other H1N1 Influenza A samples differ only in 1 nucleotide exchange. Yet, it has to be kept in mind, that only approximately 300bp of the 13.6 kbp genome is covered by the PLEX-ID primer pairs and it cannot be estimated how differences in gene compositions may affect binding of the antibody induced by the vaccination.

Sequencing of influenza samples from Phase I

To further verify results on influenza virus assay results obtained in Phase 1, sequencing of 7 influenza samples with unique amplicon compositions has been initiated. Phylogenic analysis of the influenza samples from Phase I revealed 3 H3N2 influenza signatures, with unique amplicon base pair compositions, that had no corresponding entry in the PLEX-ID database and therefore no known Genbank entry. One of the signatures was seen in 5 independent samples, whereas the other two were unique to one of the samples. These findings indicate that the genotype of these three H3N2 influenzas is unique to Hawaii and has not been described before. Yet, as the PLEX-ID primer pairs only cover up to 700bps of 1.5kbps influenza genome, whole genome sequencing was necessary to confirm the findings. To accomplish this task, cDNA is needed for sequencing, which also covered the extreme ends of the eight segments, needed to be generated. Sequencing itself is planned to be done by a third party, as TAMC does not have any in-house sequencing capabilities. cDNA generation is underway and has included so far the following steps: (1) design degenerative primers, (2) amplification of the targets with One-Step RT-PCR/PCR, (3) purification of the PCR samples with ExoSAP (exonuclease/shrimp alkaline phosphatase), (4) qualification and quantification of the PCR products with gel electrophoresis and UV spectroscopy. cDNA synthesis is almost completed and shipment to third party vendor for genome sequencing is in progress. Once sequencing is completed, genome assembly through bioinformatic tools and comparison of sequence to Genbank entries will occur to establish uniqueness of the three influenza signatures from Phase 1.

Database development

While working with the influenza dataset of 200 samples during Phase 1, it became apparent that a bigger dataset was anticipated in Phase 2 and a database would be of tremendous advantage to manage data collection and analysis. Therefore the development of a database was initiated. After reviewing the available options, Microsoft SQL Server was chosen to be used as the underlying database program. The first version of the database was available in March 2011 and has been revised and improved to even better meet the needs for data analysis. Currently the database does not only include the data points collected from the PLEX-ID, but also includes the de-identified patient information and Pathology results from TAMC. The database successfully converts the report formats from the PLEX-ID software and the DoD's CHCS clinical data management report to create a tool that allows querying for different data characteristics and sample tracking, minimizing the human error involved with data handling. In the future it would be of tremendous advantage, if the database could be developed even further to create customizable queries and reports and to expand data collection to other assays run on the PLEX-ID.

Project Progress and Plans for Publication of Results.

Overall, the outlined goals in Task 4 are approximately 85% complete at the end of September 2011, and a no cost extension has been requested and granted to perform further analyses to fully meet all of our initial objectives. To increase visibility of the newly established PLEX-ID technology at TAMC, a manuscript has been drafted for eventual publication submission. This manuscript contains the results from work done in Phase 1 Task 2 (testing of archived influenza samples), including additional data analysis for phylogenic relationships of H3N2 samples

collected and identified during Phase 1. Of the influenza samples tested, 3 have DNA base pair compositions different from any previously characterized in the IBIS flu database thus far. It appears, that these flu specimens collected from patients in Hawaii may be unique to the Pacific Region or may represent an emerging mutation of the virus. This type of detection of new virus base pair signatures is of great value to the epidemiological tracking of respiratory viruses and emerging infectious disease surveillance program we are aiming to establish in the PRMC.

KEY RESEARCH ACCOMPLISHMENTS:

- Demonstrated functionality/breadth of the RVS assay with archived samples and reference viruses
- Determined cycle time for influenza testing with the RVS assay
- Established specimen collection procedures to remove patient identifiers and perform analysis on de-identified specimens. These logistics will enable ramping up for larger numbers of specimens to be processed for future epidemiological studies. Collected and tested over 800 samples and compiled the de-identified patient data
- Analyzed data to elucidate overall virus prevalence, prevalence by age, patient category and virus patterns over 9 months. Identified multiple virus co-infections.
- Developed and deployed a functional database for storage and analysis of sample data

REPORTABLE OUTCOMES:

- PLEX-ID technology has proven to be a valuable tool for current seasonal disease surveillance of upper respiratory infections using the RVS assay.
- Analysis showed, that
    - 39% of samples collected at TAMC are positive for a virus, with 7% having more than one virus detected.
    - RSV is the most common virus detected with prevalence in children age 0-2 with most detected during October through December time frame.
    - This pattern is different from continental U.S. Additionally RSV is more likely to be associated with secondary virus detection than other viruses tested.
    - Influenza A is the second most common virus, prevalent in patients age 31 to 45. Influenza A follows the same seasonal pattern than continental U.S. Assessment of Influenza A vaccine efficiency was difficult, as only the Influenza A H1N1 genotype was present in the database,
    - Influenza B is low in frequency and has a seasonal pattern that is set off to mainland U.S. No prediction concerning the vaccine efficiency could be given.
- Testing of Pathology and PLEX-ID discrepant sample results via RT-PCR indicates that the PLEX-ID is the more sensitive method of detection as more true positive samples are identified.

CONCLUSION:

During this past year, the TAMC DCI together with SAIC and IBIS-Abbott was able to further establish the PLEX-ID as a valuable tool for disease surveillance at TAMC. We were able to establish the RVS assay for detection of upper respiratory infections and tested over 800 clinical samples routinely collected at TAMC to investigate seasonal respiratory virus patterns and dynamics. A comprehensive data set was obtained when PLEX-ID data and de-identified patient data including TAMC Department of Pathology results were merged together in the newly created SQL database. Analysis showed the multi-layered nature of the dataset, ranking from the overall epidemiological aspects to in depth understanding of virus evolution. The PLEX-ID technology and the breadth of information collected even at a genomic level is advantageous over conventional virus detection methods and makes it the ideal tool to conduct disease surveillance not only for USPACOM, but possibly for and with other DOD surveillance areas.

With Phase 2 ending, most of the goals as described in the statement of work have been met and project focus is now on the tasks described in the no-cost extension, including the testing of additional flu samples of the gap years in Phase 1 specimens and Phase 2 TAMC collections. To further understand disease pattern at TAMC the extensive samples collection from Phase 2 will be tested for bacterial co-infection, as it is strongly suspected that specimens collected from patients with clinical respiratory symptoms that tested negative for virus are likely to have bacteria colonization as the disease cause. With the development of new bacteria surveillance kits that were not available at the time of the original project proposal, these assays also need to be characterized to meet the intended overall goal of establishing the PLEX-ID technology for

Respiratory Disease Surveillance in the PRMC. Without the arsenal of available assays characterized, the capabilities of the use of the PLEX-ID at TAMC cannot be fully determined.

Additionally, more comprehensive and in depth analysis will be conducted with the data collected throughout Phase 2, including the study of each virus (such as amplicon compositions), comparison of the dataset to other DOD surveillance areas and Hawaii itself and evaluation of data for possible publication in peer reviewed journals. These scientific publications will establish and promote the PRMC PLEX-ID system for future self-sustaining funding for the overall goal of disease surveillance initiatives for the Pacific Basin.

ACRONYMS:

CDC – Centers for Disease Control and Prevention

cDNA – Complementary DNA

CHCS – Composite Health Care System

DCI – Department of Clinical Investigation

DFA – Direct Fluorescent Antibodies

DOD – Department of Defense

DNA – Deoxyribonucleic acid

hMPV – Human Metapneumovirus

HSV – Herpes Simplex Virus

LOD – Limit of Detection

PCR – Polymerase Chain Reaction

PIV – Parainfluenza viruses

PRMS – Pacific Regional Medical Command populations

RNA – Ribonucleic acid

RSV – Respiratory Syncytial Virus

RT-PCR – Reverse Transcription Polymerase Chain Reaction

RVS – Respiratory Virus Surveillance

SAIC – Science Applications International Corporation

TAMC – Tripler Army Medical Center

TOF-MS – Time-Of-Flight Mass Spectrometry

USPACOM – United States Pacific Command

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