Duquesne University

Annual Progress Report: 2011 Formula Grant

Reporting Period

July 1, 2013 – June 30, 2014

Formula Grant Overview

Duquesne University received $107,464 in formula funds for the grant award period January 1, 2012 through December 31, 2014. Accomplishments for the reporting period are described below.

Research Project 1: Project Title and Purpose

A Biomaterial Approach to Inhibit Melanoma Growth and Metastasis in Mice – The purpose of this project is to test a novel strategy that aims to neutralize transforming growth factor-beta (TGFβ), a cytokine implicated in cancer immune escape and metastasis. Antibodies have been developed to inhibit TGFβ functions in cancer patients. It was thought that anti-TGFβ antibodies could be more effective when administered directly into tumors, but studies have shown that even locally injected antibodies do not accumulate in cancer lesions. We have designed and characterized a peptide-based injectable system that can circumvent this problem. In essence, we are proposing a novel way to use antibodies to inhibit dissemination of cancer cells by decreasing local TGFβ concentration.

Anticipated Duration of Project

1/1/2012 – 12/31/2014

Project Overview

Carcinoma in mice and humans produce supra-physiological concentrations of TGFβ. In progressive cancers, elevated TGFβ enhances tumor cell invasiveness; attenuation of TGFβ signaling suppresses metastasis in mouse models of colon, skin and mammary cancers. Cancer progression driven by TGFβ is also attributed to the cytokine's ability to expand regulatory T (Treg) cells, a tumor-promoting lymphocyte, and its potent suppressive effects on cytotoxic T cells (CTLs), a type of tumor-killing lymphocytes. Thus, effective local neutralization of TGFβ should impede the growth and spread of cancer cells. The antibody displaying system is an injectable by which exceptionally high concentrations of anti-TGFβ antibodies can be retained in tumors.

The objective of the project is to demonstrate in vivo functions of a novel multivalent anti-TGFβ antibody assembly. Underpinning the research is the engineering of self-assembling

peptides to form a gel-like substance to display IgG molecules in cluster. The amphiphilic peptide AEAEAKAKAEAEAKAK (single amino acid code; hereafter "EAK") self-assembles to form stable β-sheet fibrous networks in vitro and in vivo. We have previously reported a design in which the EAK network is modified to bind and orient multiple immunoglobulin-gamma (IgG) molecules through consecutive histidines (his-tag). Recent preliminary data show that IgG molecules form clusters in tumors for at least 24 hours, at which time free antibodies were no longer detectable. These data unequivocally demonstrate the ability of the system to enhance local concentration of antibodies in tumors. What is not known is the extent to which anti-TGFβ clusters can impede TGFβ functions in tumors.

The hypothesis is that clustering of anti-TGFβ IgG enhances the neutralization of the cytokine in vivo. The study entails injecting anti-TGFβ clusters into established syngeneic melanoma tumors in mice. We will investigate in tumors TGFβ concentration and signaling, frequency of Tregs recovered from tumors, and metastasis of cancer cells to draining lymph nodes. Changes in these outcomes will be measured with respect to dose and frequency of administration.

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Expected Research Outcomes and Benefits

Tumor-derived TGFβ is a critical mediator in cancer growth and metastasis. Currently, no anti-TGFβ therapies have been approved in the U.S. This project seeks to advance a tool by which drug delivery barriers in solid tumors can be circumvented. Systemically injected antibodies do not accumulate in tumors to a great extent. Intratumoral injection can bypass the vascular barriers, but nonetheless drug molecules rapidly leave from the site of injection. We have devised a peptide-based scaffold on which IgG molecules can be concentrated and spatially oriented. Preliminary studies show that antibodies displayed by the system remain in living tumors much longer than those injected without the system. The research will generate data to determine the extent to which the displayed antibodies can neutralize TGFβ in the tumor microenvironment. We will identify the optimal dosing regimens and benchmark against conventional intravenous administration. The clinical significance of the research is that it can lead to new treatment modalities for suppressing dissemination of localized breast and skin tumors. The injectable anti-TGFβ clusters can reduce the metastatic potential of cancer cells in patients awaiting surgery. It can be applied after tumor resection to generate a local antitumor immunity to decrease the likelihood of relapse. It may provide an option for patients with non-

resectable tumors. The anti-TGFβ clusters can be used as an adjuvant for cancer vaccines by diminishing Treg cells and unleashing tumor-specific CTLs. The potential impact is high because the system can be adapted to antibodies already in clinical development.

Summary of Research Completed

In addition to tumor growth rate, phenotypic change is a relevant measure of TGFβ-driven malignancy. We investigated the effects of the anti-TGFβ clusters in reversing epithelial-mesenchymal transition (EMT) in a solid tumor model. EMT is a complex process in which pre-malignant epithelial cells lose their cell-cell adhesion and become mesenchymal-like cells with migratory and invasive properties.

We postulated that antibodies injected in saline would be cleared from the tumor mass more rapidly than antibodies injected via the system. The extended duration of the antibodies inside the lesion would translate into an impact on the level of TGFβ. An assumption is that the antibodies displayed by the system remained in their native and active conformation.

The experiment entailed inoculating BALB/c mice with 4T1 cells, a syngeneic mammary line. Anti-TGFβ (5 μg per tumor) with ("system") or without ("control") the self-gelling materials were injected into small tumors (when diameters reached ~3 mm) (Figure 1). After three injections, tumors were harvested and mRNA was extracted.

Transition to mesenchymal phenotypes was determined using real-time PCR analyzing an array (SuperArray Biosciences) of genes implicated in EMT. The array contains 84 genes known to govern apical to basal-lateral polarity (e.g. Smad2, Snai2, Notch1), intercellular tight junctions (CDH2), acquisition of abilities to degrade ECM (e.g. matrix metalloproteinases) and motility (e.g. Rho) [#4768]. Up- or down-regulation of these genes are indicative of metastatic potential, thereby providing functional measures of TGFβ inhibition.

The data appear to indicate that cadherin molecules (Cdh1 and Cdh2) were upregulated in tumors treated with clustered anti-TGFβ compared to antibodies in saline (Figure 2). This outcome was consistent with the notion that the pro-metastatic effects of TGFβ were reversed by the clustered antibodies. The increase in collagen deposition in the tumors may reflect extracellular matrix remodeling, a sign of fluctuating TGFβ level. Interestingly, no difference was observed in the expression of Smad2, a signaling molecule associated with TGF receptors upstream of cell adhesion regulation and other TGFβ-driven phenotypic changes. The timing of the assay may mismatch with the kinetics of Smad expression due to the transient flux of TNFR signaling.

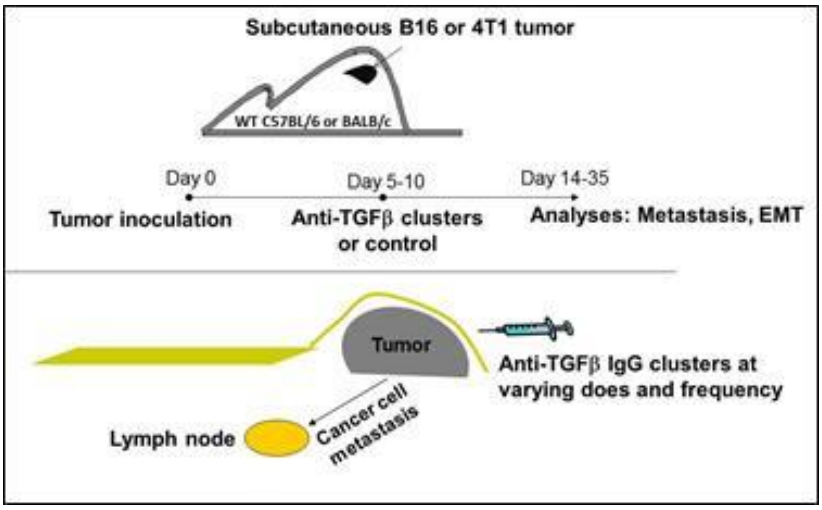
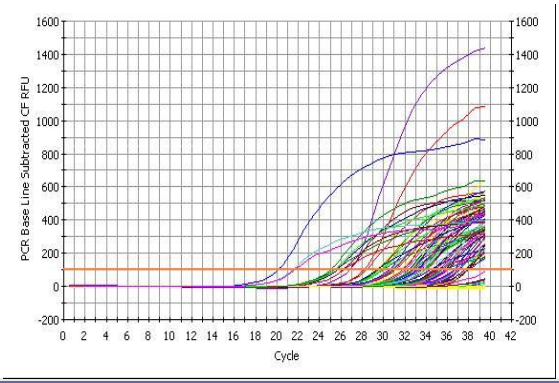
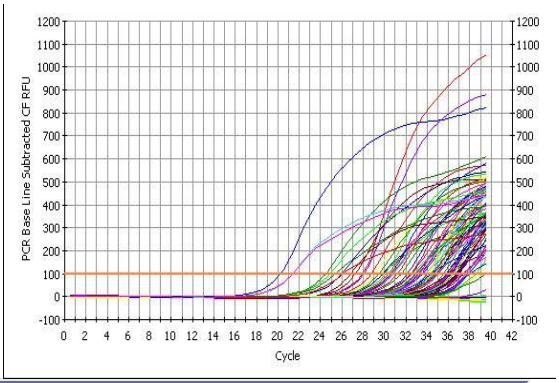


Figure 1. 4T1 tumors were established subcutaneously in BALB/c mice with 10^4-10^6 cells. Clustering or free antibodies will be injected in a small volume (50 µl) of deionized sterile water into medium size tumors. Only tumors of equivalent size (~3 mm±0.3mm) were used and animals with ulcerating lesions will be excluded from the studies.





Array Layout

Ahnak A01	Akt1 A02	Bmp1 A03	Bmp7 A04	Cald1 A05	Camk2n1 A06	Cav2 A07	Cdh1 A08	Cdh2 A09	Col1a2 A10	Col3a1 A11	Col5a2 A12
Ctnnb1 B01	Dsc2 B02	Dsp B03	Egfr B04	Erbb3 B05	Esr1 B06	F11r B07	Fgfbp1 B08	Fn1 B09	Foxc2 B10	Fzd7 B11	Gng11 B12
Gsc C01	Gsk3b C02	Igfbp4 C03	Il1rn C04	Ilk C05	Itga5 C06	Itgav C07	Itgb1 C08	Jag1 C09	Krt14 C10	Krt19 C11	Krt7 C12
Mitf D01	Mmp2 D02	Mmp3 D03	Mmp9 D04	Msn D05	Mst1r D06	Mtap1b D07	Nodal D08	Notch1 D09	Nudt13 D10	Ocln D11	Pdgfrb D12
Plek2 E01	Pppde2 E02	Ptk2 E03	Ptp4a1 E04	Rac1 E05	Rgs2 E06	Serpine1 E07	Sip1 E08	Smad2 E09	Snai1 E10	Snai2 E11	Snai3 E12
Sox10 F01	Sparc F02	Spp1 F03	Stat3 F04	Steap1 F05	Tcf7l1 F06	Tcf4 F07	Tfpi2 F08	Tgfb1 F09	Tgfb2 F10	Tgfb3 F11	Timp1 F12
Tmeff1 G01	Tmem132a G02	Tspan13 G03	Twist1 G04	Vcan G05	Vim G06	Vps13a G07	Wnt11 G08	Wnt5a G09	Wnt5b G10	Zeb1 G11	Zeb2 G12
Gusb H01	Hprt H02	Hsp90ab1 H03	Gapdh H04	Actb H05	MGDC H06	RTC H07	RTC H08	RTC H09	PPC H10	PPC H11	PPC H12

Figure 2**.** qPCR results generated from 4T1 tumors injected with anti-TGFβ antibodies delivered in self-assembling components or in saline. mRNA samples were profiled using the Mouse EMT RT² Profiler™ PCR Array on a BIO-RAD iCycler (iQTM Optical Module).

Amplification cycles			
	System	Control	Δ
Col1a2	31.84	33.91	–2.1
Cdh1	33.85	36.04	–2.2
Cdh2	32.63	34.89	–2.3
Col3a1	34.40	38.33	–3.9

Research Project 2: Project Title and Purpose

Determination of the Role of pilin glycosylation in Pseudomonas aeruginosa Infections – The purpose of this project is to discover new knowledge concerning the distribution, among clinical isolates, of *Pseudomonas aeruginosa* strains producing glycosylated pili. This organism is a major cause of hospital-acquired pneumonia. We have found that *P. aeruginosa* strains producing glycosylated pili are associated with acute pneumonia. Respiratory tract damage caused by smoking includes a greatly increased susceptibility to pneumonia. The information gained from this project can be applied to the prevention or treatment of acute pneumonias caused by this organism. Prevention would be through vaccine design based on pilus structure. Treatment would be through the development of chemotherapeutic agents that interfere with pilin glycosylation.

Anticipated Duration of Project

1/1/2012 – 12/31/2014

Project Overview

The broad objective of this research project is to establish the relationship between the group I pilin glycosylation state in *P. aeruginosa* and specific diseases caused by this organism. A specific aim of this project will be to do a comprehensive survey of the group I pilin glycosylation state of *P. aeruginosa* clinical isolates representing acute disease (such as pneumonias, urinary tract infections, burn infections, keratitis, ear infections, folliculitis and septicemias), as well as chronic infections such as those occurring in patients with cystic fibrosis or COPD (chronic pulmonary obstructive disease). This effort will first require the establishment of a comprehensive collection of clinical *P. aeruginosa* isolates with representative numbers of each of the diseases mentioned. These will be obtained from collaborators at local hospitals and institutes in Pittsburgh as well as elsewhere. The determination of group I pilin distribution will be accomplished by Western blot using a group I pilin-specific monoclonal antibody. A second aim is to do a complete survey of the O-serotype of all isolates collected. This will be done by serotyping and will allow the determination of O-antigen frequency among strains producing group I pilins. Finally, the laboratory results obtained will be subjected to a rigorous statistical analysis frequency of group I glycosylation and non-glycosylation will be compared between various infections sites. P values will obtained using 2x2 contingency tables and employing The Fisher's exact test. A two tailed P-value calculation will be used.

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Other Participating Researchers

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Expected Research Outcomes and Benefits

My hypothesis is that the pilin glycan is important to the outcome of specific diseases caused by *P. aeruginosa*. Preliminary evidence suggests that this is the case with acute respiratory disease for which this organism is the etiological agent. Work presented will confirm or reject this hypothesis and will also examine other diseases caused by this organism to determine whether group I pilin glycosylation influences the distribution and frequency of this trait. This information could have practical value. Determination of a tropism caused by a particular pilin type and by an O-antigen serotype will allow for prophylaxis or chemotherapy.

Group I pili are very homogeneous in sequence and are extremely immunogenic. It has previously been shown that *P. aeruginosa* pili are protective against challenge from whole cells by a respiratory route. The lipopolysaccharide O-antigen of this organism has been shown to be an excellent vaccine target. While the O-antigen of *P. aeruginosa* is much more structurally variable than the pilin of this organism, three common serotypes predominate. In addition, we have shown that the glycan of group I pilin produces a protection which targets the O-antigen. A group I pilin vaccine preparation containing major O-antigen representatives would be expected to provide broad protection.

While active immunization of individuals at risk for these respiratory infections is an important goal, passive immunization of individuals in critical condition is also a consideration. Future work in this area would require human monoclonal antibodies directed against group I pilin or against the glycan. Antibodies directed against the pilin glycan would inhibit twitching motility, a trait required for virulence, and also stimulate opsonization.

Summary of Research Completed

Introduction: The first goal of the project was strain isolation. A total of 208 clinical *Pseudomonas aeruginosa* isolates have been received from Children's Hospital while 37 strains of this organism were previously collected from Mercy Hospital. Both are University of Pittsburgh Medical Center-affiliated organizations. I have decided to concentrate on the Children's Hospital isolates for the remainder of the grant period since I have more information (date of isolation, concrete information on infection site) on these strains.

The type IVa pili are protein fibers extending from the surface of many Gram-negative bacteria. This structure is vital to pathogenicity in that it facilitates adhesion to biotic and abiotic surfaces and mediates a form of surface motility called twitching. The type IVa pili of *P. aeruginosa* can be placed into several groups based on pilin (the monomeric subunit of the pili) primary structure. Group I pilin is noteworthy in that it is glycosylated, having an O-antigen repeating unit attached to the β-hydroxyl group of the C-terminal serine.

The previous Annual Progress Report described the development of a polymerase chain reaction protocol which allowed screening of isolates for the presence the gene coding for group I pilin. These results were confirmed by Western blot using a monoclonal antibody specific for group I pilin. The Western blot also allowed for the determination of the pilin glycosylation state. Using these methods we were able to place all hospital isolates into those capable of producing either group I or non-group I pilin.

Frequency and analysis of group I pilin glycan among clinical isolates: The second goal of this project was to determine the frequency and distribution of O-antigen repeating units associated with group I pili. The most widely accepted serotyping protocol for *P. aeruginosa* is the International Antigen Typing System (IATS) which is based on the O-antigen. This system detects 20 serotypes by agglutination, but is probably not comprehensive. Another disadvantage is that it is expensive which makes screening of a large number of samples impractical. A further drawback is that serotyping does not give structural information concerning any specific pilin glycan. With this in mind we used a different approach to determining the O-antigen distribution among group I and non-group I strains. Work by Raymond and co-workers showed that O-antigen biosynthesis gene clusters for all of the IATS serotypes fell into 11 sequence families. While certain clusters produced several serotypes, each produced a saccharide that contained shared fundamental characteristic sugars, linkages, and modifications. We refer to these clusters as Raymond (or R-) types. Examination of these gene clusters showed us that each contained unique 3'-proximal sequence. With this in mind, specific primers for each of the 11 R-types were prepared and tested using standard *P. aeruginosa* strains producing known IATS serotypes for reference.

The results presented in the 2013 progress report were preliminary due to the incomplete typing of these strains. During the current grant year, we have reconstructed some of the oligonucleotide primers and have refined amplification conditions. We are now able to give a much more complete and unambiguous description of the frequency and distribution of these R-types. Figure 1 shows that while the entire collection of group I strains could be typed, 5 of the 97 non-group I strains were nontypable. No strains were polytypable. Again, the R5 type is clearly dominant in the whole population. In agreement with our original results, re-examination of the strains using the 2X2 contingency table analysis showed that R5 was found much more frequently ($p < 0.0001$) among strains producing group I pili. On the other hand, both R2 and R9 types were rarely found in the group I strains but were well represented among the non-group I isolates ($p < 0.0001$ in both cases). Our re-evaluation of these strains no longer showed a significant difference of R3 type between the two pilin groups.

Correlation of strain frequency with disease site: Each clinical isolate studied was placed into one of three infection site groups (chronic respiratory, acute respiratory, and non-respiratory)

based on information provided by the source Hospital. The distribution of R-types among isolates from these disease sites is presented in figure 2.

The R5 group was examined more closely since it possessed large enough isolate numbers to potentially give statistically significant results. Figure 3 shows that there is no correlation between this R-type and any of the three disease types. Further, there is no statistical significance found in comparing group I pilin producers with disease group (Figure 4).

However, an association between R5 and group I pili was detected in the acute respiratory group. The random chance of an R5 strain producing group I pili was calculated to be 22.3%. The frequency of these two phenotypes existing together among isolates from chronic respiratory disease was 25.6%, while this correlation among strains from non-respiratory sources was 31.2%. Comparing these frequencies with chance distribution using the 2X2 contingency table analysis showed that neither ($p = 0.5381$ and 0.1234 respectively) were significant (Figure 5). The frequency of R5 type among strains producing group I pili within isolates from acute respiratory infections, however, was 47.7% resulting in a p value of 0.0011 which is statistically very significant. This finding suggests that *P. aeruginosa* strains producing both the R5 O-antigen repeating unit and glycosylated group I pili are more likely to cause acute respiratory infections. These results are particularly interesting because this study has shown that both the R5 O-antigen type and the group I pilin type are dominant in *P. aeruginosa* clinical populations. My original hypothesis was that glycosylated group I pili were more frequently found among acute pneumonia infections. The actual results suggest a more specific response, that strains glycosylated with the R5 glycan are specifically associated with this type of infection.

Since the reservoir for *P. aeruginosa* infections is natural environments (soil, water, plant material), the high frequency of R5 O-antigen and group I pilin in the whole isolate pool predicts that these types are predominant in the strains coming in contact with vulnerable individuals (often patients in the hospital environment). However, the high frequency of group I/R5 strains among patients with acute respiratory disease suggests a specific susceptibility pattern. This also suggests an intervention strategy for dealing with acute respiratory infections in which targeting either group I pilus type or R5 O-antigen type could result in reducing the morbidity resulting from these infections. This could be the form of pilus based immunization either in the form of an active vaccine or the generation of passive immunity with the use of immune serum. In addition, targeting steps in group I pilin biosynthesis (glycosylation for example) or R5 O-antigen repeating unit for chemotherapy would be a promising approach.

There was no statistical significance between other R types and the disease groups as determined by 2X2 contingency table (results not shown). In addition, there was no correlation between pilin type and these disease groups (results not shown).

<u>Molecular basis for group I pilus glycan selectivity</u>: The distribution of R-types among strains producing group I pilin suggests the selection of glycan oligosaccharide. This could occur through the specificity of PilO, the oligosaccharide transferase responsible for pilin glycosylation. This is not likely since evidence from my laboratory has indicated that this enzyme has a very broad specificity. More importantly, we have previously shown that glycans from either of the snubbed R-types (R2 and R9) can be attached to pilin when expressed from a

plasmid carrying a functional *pilO* gene. An alternative possibility is that these glycans are not equally effective in supporting pilus function. We have evidence that the pilin glycan influences pilus solubility by attenuating hydrophobicity of this structure. We have also shown that the non-glycosylated pilus produces markedly lower twitching motility at ionic strengths commonly found at *P. aeruginosa* disease sites. If the glycan has this role, I suggest that the R-types influence the group I pilus function to varying degrees based on oligosaccharide structure.

All R-types are composed of either three or four sugars. Figure 6 shows the structure of the three R-types which are either preferred (R5) or selected against (R2 and R9). It can be seen that the R5 structure is much larger due to the fact that it is composed of four, rather than three, sugars. A comparison of the sugar number distribution among strains producing group I and non-group I pilins is shown on Table I. Here it can be seen that the oligosaccharides composed of four sugars are found primarily among strains producing group I pili. The non-group I strains more frequently produce glycans containing three sugars. This comparison is highly significant having a p value of less than 0.0001 as determined using the 2x2 contingency table. This strong relationship is found among each of the three disease types.

We have, through several lines of evidence, recently shown that production of only non-glycosylated group I pilin strongly reduces piliation. For example, a mutant of *P. aeruginosa* strain A074 (one of the Childrens Hospital isolates producing group I pilin) which was engineered to contain a deletion in the *pilO* gene produced only non-glycosylated pilin and markedly reduced piliation. Complementation of this strain with a functional *pilO* gene resulted in both pilin glycosylation and normal piliation. Further, expression of the A074 *pilA* gene in ***P.*** *aeruginosa* strain 1244.47, a mutant strain with an inactive pilin gene, produced low levels of piliation. Expression of a functional *pilAO* operon in this strain resulted in active piliation.

It is possible that group I pili are not found in strains producing R2 and R9 O-antigen because such strains are selected against due to their production of less effective pili. We will test this hypothesis during the next six months by expressing a functional cloned *pilA* gene or *pilAO* operon in *P. aeruginosa* strains producing the R2 or the R9 oligosaccharide. The strains used will be PAO1 and PA103. Strain PAO1 produces an R2 O-antigen repeating unit while that synthesized by PA103 is R9. Since the production of host strain pilin might interfere with piliation from heterologous genes, mutants of these strains having a pilA defect will be obtained from researchers in the field.

It is further possible that strains producing R5/group I pili are more resistant to respiratory-specific host defense mechanisms. These could include the response of alveolar macrophages or complement fixation in the respiratory environment. This important area of work is beyond the scope of the remaining grant time, but will be the focus of future efforts.

Publication of research and future studies. Results are currently being prepared for publication. In addition, this work has opened up the possibility of future studies and will be the basis research grant proposals.

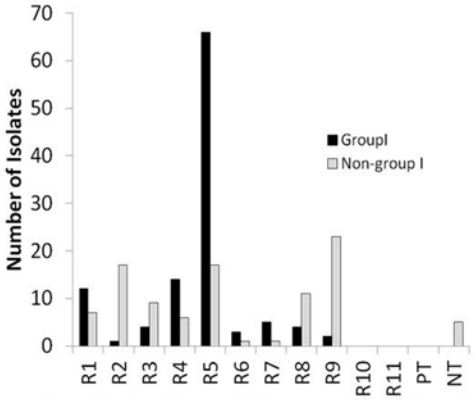

Figure 1. Frequency of R-types among *P. aeruginosa* isolates producing group I or non-group I pilin.

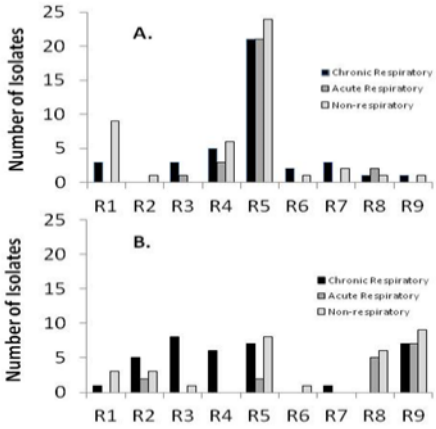

Figure 2. Distribution of R-types among disease groups. **A.** Strains producing Group I pilin. **B.** Strains producing non-group I pilin.

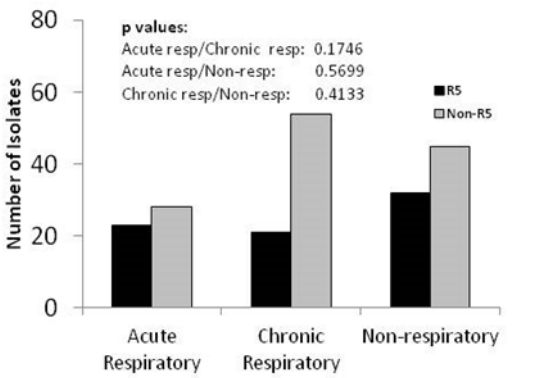

Figure 3. Comparison of R5 frequency among disease groups.

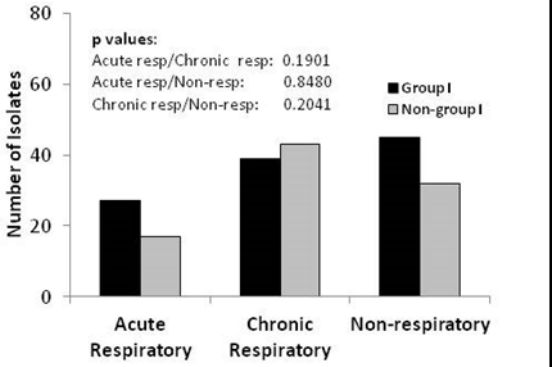

Figure 4. Comparison of group I pilin type frequency among disease groups.

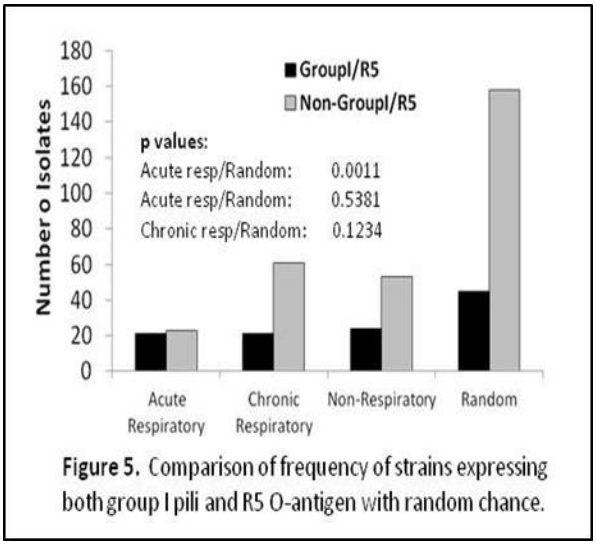

Figure 5. Comparison of frequency of strains expressing both group I pili and R5 O-antigen with random chance.

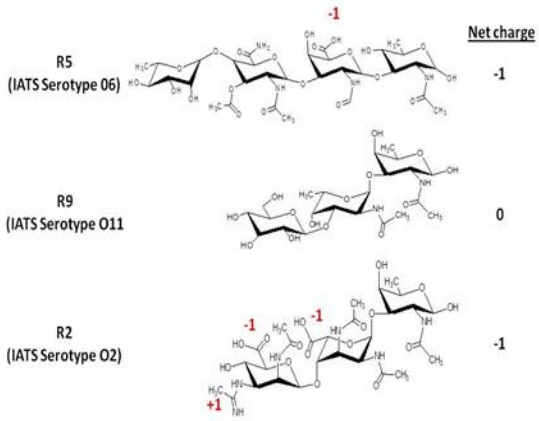

Figure 6. Structure and charge of examples of R5, R9, and R2 O-antigen repeating unit structure. International Antigenic Typing Scheme (IATS) examples (ref) are presented.

Table I. Comparison of distribution of O-antigen sugar number of group I and non-group I strains from three disease groups

	Group I Strains		Non-group I Strains	
	3 sugar	4 sugar	3 sugar	4 sugar
Chronic respiratory	7	33	18	25
Acute respiratory	2	25	15	2
Non-respiratory	6	39	20	12
Total:	15	97	53	39

$P < 0.0001$