CAPSULAR POLYSACCHARIDE RELEASE BY SEROTYPE 3 PNEUMOCOCCAL STRAINS REDUCES THE PROTECTIVE EFFECT OF ANTI-TYPE 3 CAPSULAR ANTIBODIES

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The efficacy of the serotype (ST) 3 pneumococcal component of the conjugate vaccine (PCV) remains unclear. While the CPS synthesis of most serotypes is wzy-dependent, strains of two serotypes, 3 and 37, synthesize CPS by the synthase-dependent pathway, resulting in polysaccharide that is not covalently linked to peptidoglycan and can be released during growth. We hypothesized that release of CPS during growth reduces anti-type 3 CPS antibody-mediated protection and may explain the lower efficacy of the type 3 component of PCV. Mice were injected with capsule-specific antibodies and challenged intraperitoneally (IP) with or without addition of sterile culture supernatant containing type-specific CPS. In vitro released CPS concentrations per 10^7 CFU of ST3 and 37 strains were significantly higher than for ST 1, 4, 6B, and 14 strains. Following IP injection in mice, blood concentrations of CPS were significantly higher for the ST3 than for ST4/5 strains. Opsonophagocytic killing assay (OPKA) titer of anti-type 3 CPS antibody was significantly reduced by type 3 CPS, culture supernatant or serum from ST3 WU2-infected mice. In IP challenge, the addition of 0.2 µL of culture supernatant from WU-2 inhibited passive protection, whereas 100-fold more culture supernatant from ST4 strain TIGR4 was required for inhibition of protection. We conclude that released type 3 CPS interferes with antibody-mediated killing and protection by anti-CPS antibody. The relative failure of ST3 PCV may be due to CPS release, suggesting that alternative immunization approaches for ST3 may be necessary.
Streptococcus pneumoniae remains a major cause of bacteremia, meningitis, pneumonia, and acute otitis media worldwide (1). For the 94 pneumococcal serotypes (ST) that have been identified, the synthetic mechanisms of capsular polysaccharide (CPS) can be classified into two pathways: a synthase-dependent and a wzy-dependent pathway (2-5). A major difference between the two pathways is that wzy-dependent synthesis results in CPS that is covalently linked to the peptidoglycan on the bacterial cell wall whereas the synthase-dependent CPS is bound on phosphatidyglycerol or the synthase on the membrane (2). Therefore, synthase-dependent serotypes can release CPS either by dissociation from phosphatidyglycerol or by ejection from the synthase (6); this release can be detected in vitro as shown in (7).

While the CPS synthesis of most serotypes is wzy-dependent, strains of two serotypes, 3 and 37, synthesize CPS by the synthase-dependent pathway (3, 8). Whereas ST37 strains are rarely isolated from humans, ST3 isolates are an important cause of invasive pneumococcal disease, particularly pneumonia in both children and adults (9). With the expansion of the 7-valent to the currently used 13-valent pneumococcal conjugate vaccine, serotype 3 conjugate was added to the formulation. The immunogenicity of the type 3 conjugate led to the expectation that both colonization and infection with strains of this serotype would decline significantly, as has been noted for the other serotypes included in pneumococcal conjugate vaccines. Surprisingly, however, there have been conflicting reports on the efficacy of the serotype 3 component of the pneumococcal conjugate vaccine (PCV) (9-11).
Despite predicted effectiveness at the accepted 0.35 µg/ml ELISA cutoff of 97% (11), to date it does not appear that PCV13 has resulted in the same degree of reduction in incidence of type 3 disease as seen with other newly included serotypes, such as 19A.

We hypothesized that the unusual polysaccharide synthesis pathway of ST 3 strains may provide an explanation for these findings. To evaluate this, we first compared CPS release in vitro and in vivo by various serotypes and sought to determine whether the amount of released CPS from ST3 pneumococci is sufficient to inhibit antibody-dependent bacterial killing and protection against serotype 3 pneumococci.
METHODS

Bacterial strains and Reagents

Purified pneumococcal CPS of various serotypes were purchased from ATTC; these were used for all vaccine preparations and ELISA. Pneumococcal strains used in this work are listed in Table 1. All strains were grown in Todd-Hewitt broth containing 0.5% yeast extract (THY) at 37°C with 5% CO₂ until optical density at 600 nm (OD₆₀₀) of 0.5 was reached. Bacterial stocks were stored in THY medium with 20% glycerol at -80°C until future use. A CPS-deleted (ΔCPS) ST3 strain was generated by deleting the operon that contains two genes, cps 3D and cps 3S which are essential for serotype 3 CPS biosynthesis (12) using the Janus cassette strategy (13). A ΔCPS TIGR4 strain was also generated by deleting the whole cps gene cluster (14). Capsule deletion was confirmed by PCR, by colony appearance on blood agar plates, by the Quellung reaction (Statens Serum institute Denmark), and/or by inhibition ELISA.

To generate a large volume of sera against ST3, ST4 or ST5 capsule, rabbits were immunized with affinity-coupled complexes of target CPS, using the Multiple Antigen Presentation System (MAPS) technology we described previously (15). Briefly, ATCC ST3, ST4 and ST5 CPS were activated by 1-cyano-4-dimethylaminopyridinium tetrafluoroborate (CDAP) and then labeled with EZ-Link Amine-PEG3-Biotin (Pierce). Free EZ-Link Amine-PEG3-Biotin was removed by dialysis. MAPS complexes were assembled by incubation of biotinylated CPS with purified egg white avidin (Sigma) at room temperature overnight and then purified by
size-exclusion chromatography (SEC). Mice sera against multiple pneumococcal
polysaccharides were generated by immunizing mice subcutaneously three times at
a two-week interval with 2/5th the adult dose of PCV13 (Pfizer). Mouse sera against
ST37 capsule were generated by subcutaneous immunization with MAPS complex
made from biotinylated ST37 CPS and egg white avidin.

CPS-specific IgG antibodies were measured by ELISA using endpoint titer. 96-well
ELISA plates were coated with purified CPS of various serotypes (ATCC) according
to the conditions described in WHO instruction of Pneumococcal Serology, or the
conditions optimized in our laboratory. Briefly, ELISA plates were incubated with
purified CPS at 2 μg/ml for type 1, 1 μg/ml for type 3, 0.5 μg/ml for type 4, 10 μg/ml
for type 6B, 1 μg/ml for type 14 and 0.5 μg/ml for type 37 at 37 °C for 5 hours and
then at 4 °C overnight before use. Endpoint titer was defined as the highest dilution
of sera that did not give any signal. All sera were heat-inactivated before use in
killing assays or passive immunizations.

**Measurement of released CPS concentration following in vitro growth**

A frozen aliquot was thawed and incubated in THY medium at 37°C with 5%CO2
until OD600 0.8 was reached. Bacterial colony forming unit (CFU) was determined
by plating dilutions on blood agar plates. Bacteria-free culture supernatant was
collected by centrifugation for 10 min at 13,000 rpm followed by filtration through a
0.65 μm filter (Millipore, Billerica, MA); sterility was confirmed by plating an aliquot on
blood agar plate. To measure the CPS on the bacterial surface, the bacterial pellet
was washed with PBS (pH 7.4) and fixed with 4% paraformaldehyde at 4°C
overnight. The amount of CPS on the bacterial surface or in the culture supernatant was measured by inhibition ELISA using sera from mice immunized with PCV13, for type 1, type 3, type 4, type 6B and type 14, or sera from mice immunized with MAPS complex for type 37. A standard curve of inhibition was generated by the addition of different concentrations of purified CPS (range from 0.1 to 100 μg/mL). For each serotype, the dilution of anti-sera was optimized according to the detection range of the standard curve. Inhibition of anti-CPS antibody binding to the plate by different dilutions of bacterial supernatant or fixed pneumococci was evaluated against the standard curve. The concentration of CPS in the supernatant or on the bacterial surface was then normalized by the CFU count of the culture.

**Opsonophagocytic killing assay (OPKA)**

OPKA was performed using the protocol described by Romero-Steiner et al. (16). Briefly, HL60 cells (ATCC, Manassas, VA) were propagated in Iscove’s Modified Dulbecco’s Medium supplemented with 20% fetal bovine serum (FBS) and then differentiated into granulocytes by the addition of 100 mM dimethylformamide for 5 days. Heat-inactivated immune rabbit sera were diluted with Hanks balanced salt solution (HBS, Corning Cellgro) containing 10% FBS. Frozen bacteria stock was thawed, washed and resuspended with Hanks balanced salt solution containing 10% FBS. To determine the OPKA titer, 10 μL of bacteria (1000 CFU) was incubated with 20 μL of serial dilutions of rabbit sera in a 96-well microtiter plate, for 30 min at room temperature, and shaken at 600 rpm. Forty μL of differentiated HL60 cells (4x10^5 cells) and 10 μL of baby rabbit complement were then added (final volume, 80 μL per well) and the mixture was incubated at 37°C for 45 min with shaking. After incubation,
5 μL of mixture were plated onto a blood agar plate in triplicate. Blood agar plates were incubated for 6-8 hrs at 37°C with 5% CO₂ then placed at room temperature on ambient air overnight to facilitate colony counting of the serotype 3 strains, which are very mucoid. The highest serum dilution that resulted in 100% killing was selected for OPKA inhibition analysis.

To measure the inhibitory effect of released type 3 CPS on bacteria killing, OPKA was performed with the addition of culture supernatant or mouse sera obtained at 24 hours following IP injection with 10³ CFU of WU-2 strain. In each well, 10 μL of diluted rabbit sera were mixed with 10 μL of WU-2 strain (1000 CFU) and 10 μL of culture supernatant or mouse sera for 30 min at room temperature before HL-60 cells and complement were added. An inhibitory standard curve was generated by adding different amounts of purified type 3 CPS using either ΔCPS WU2 culture supernatant or naïve mouse sera as the diluent. The percent inhibition of in vitro bacterial killing was compared to the OPKA with the addition of ΔCPS WU2 culture supernatant or naïve mouse sera containing no purified CPS. Experiments for the determination of OPKA titer and % inhibition of killing were each repeated at least three times.

Measurement of CPS release in mouse blood following intraperitoneal infection

Female 6- to 8-week old C57BL/6J mice were used (Jackson Laboratories, Bar Harbor, ME) for all animal works. All animal protocols were approved by the Boston Children’s Hospital IACUC. Intraperitoneal (IP) infection was performed with 10³ CFU of ST3, ST4 or ST5 strain in a 200 μl volume. Mice were sacrificed 24 hours after
infection. Blood samples were plated for CFU count. Serum was then collected and filtered through 0.65 μm filter to remove live pneumococci. The concentration of CPS in serum was measured by inhibition ELISA as described above, and normalized by the CFU count in the blood.

**Passive protection and intraperitoneal challenge in mouse model**

To evaluate the extent to which released CPS could interfere with passive protection conferred by anti-capsular antibodies, the amount of serotype-specific anti-CPS antibody necessary to confer 80-100% protection against IP challenge by 10³ CFU of WU-2 or TIGR4 strain was determined. The antibody was then administered to mice 24 hours before IP challenge. For the challenge, pneumococci were re-suspended in the culture supernatant of a ΔCPS strain with or without the addition of different volumes of culture supernatant derived from growth of encapsulated WU2 or TIGR4 strain. Mouse survival was monitored daily for 7 days after IP challenge. In this passive protection challenge model, the inhibitory potential of culture supernatant was calculated by comparing the survival rate between mice that received the challenge strain diluted in supernatant from the ΔCPS mutant alone vs. supernatant from the ΔCPS strain to which supernatant purified from the growth of encapsulated strains was also added.

**Statistical analysis**

The differences in CPS concentration in culture supernatant and mouse sera by serotype were analyzed using the Mann Whitney U test. Differences in inhibitory effects of supernatant on mouse survival were by the log rank test. For all comparisons, P<0.05 was considered to represent statistical significance. All
statistical analyses were performed using PRISM (version 5.0d).
RESULTS

CPS release and bacterial content following in vitro growth by different serotypes

It has been known for some time that the type 3 polysaccharide in S. pneumoniae cultures can be found both associated with the bacterial cells and also soluble in the culture medium (17), but the extent to which this occurs compared to other serotypes has not been fully explored. We were also interested in seeing whether this release of PS in the medium would occur with type 37 strains, in which capsular polysaccharide synthesis is also synthase-dependent. Here different strains representative of the two synthetic pathways (synthase-dependent, 3 and 37; wzy-dependent, 1, 4, 6B, and 14, see Table 1) were grown in THY and supernatants harvested. Serotype-specific CPS concentration in the bacteria-free culture supernatant and on the bacterial pellet was measured by inhibition ELISA. Mean released CPS per $10^7$ CFU of ST 3 (60 µg) and 37 (130 µg) strains were significantly higher than ST 1, 4, 6B, and 14 (0.4–10 µg) (Fig. 1). The ratio of CPS released into the culture supernatant to that present on bacterial surface was greater than 1 for synthase-dependent serotypes whereas it was less than 1 for wzy-dependent serotypes (Fig. 1).

CPS release in blood following IP challenge

Next we wished to examine whether PS release from the bacterial cell could also be detected during experimental bacteremia in mice. Mice were infected with strains of serotype 3, 4, or 5 at a dose of $10^3$ cfu IP (n=5 mice per group). Bacteremia was documented in all mice at 24 hours following IP challenge. CFU counts in blood...
ranged from $6 \times 10^4$ CFU/ml to $1.3 \times 10^8$ CFU/ml depending on the serotype of the infecting strain (Fig. 2A). At 24 hours following IP injection, the median serotype 3 CPS release in blood per $10^7$ bacteria was 31.2 μg, an amount that is significantly higher than that seen following infection with a strain of serotype 4 (<0.1 μg, P=0.016) or 5 (0.8 μg, P=0.008) (Fig. 2B).

**Inhibition of bacterial killing in vitro by the addition of mouse sera or filtered culture supernatant**

To evaluate the impact of released type 3 CPS in vitro or in vivo on the antibody-mediated killing of pneumococci, we performed OPKA against WU2 strain in the presence of culture supernatant or mouse sera post WU2 infection. We first determined the minimal amount of anti-type3 CPS rabbit serum that would be sufficient to achieve between 90-100% bacterial killing in a standard OPKA (Fig. 3A). This amount of antibody was then used in further assays to measure inhibition of bacterial killing. A standard inhibition curve was developed by the addition of purified type 3 CPS (diluted with the culture supernatant of ΔCPS WU2 strain) ranging from 0.15 to 150 ng. About 50% inhibition of bacterial killing was observed when 12 ng of purified type 3 CPS was added to the OPKA (Fig. 3B, solid line). A similar inhibition capacity was observed with the culture supernatant of WT WU2 strain (containing 150 μg/mL of released type 3 CPS). As shown in Fig. 3B (dashed line), the bactericidal activity of anti-serotype 3 antibody was significantly reduced in the presence of WU2 culture supernatant in a CPS concentration-dependent manner.

Next, we compared the killing activity of anti-type 3 CPS antibody in the presence of naïve mouse sera (defined as 100% killing) or mouse sera (from n=5 mice) following
IP challenge with WU2 strain. As shown in Fig. 3C, addition of 30 µL of mouse sera post-IP infection with WU2 (containing 0.4-0.7 µg/mL of released type 3 CPS) showed 26%-52% inhibition of bacterial killing, comparable to what could be observed when an equivalent amount of purified CPS was added.

**In vivo inhibition of protection in passive transfer-IP challenge model**

Next, we wished to compare the relative capacity of bacterial culture supernatants from type 3 or 4 strains to inhibit protection by passive transfer of anti-capsule antibodies in an intraperitoneal sepsis model. First, we determined the minimum amount of capsule-specific rabbit sera that provided >80% protection against IP challenge: 40 and 20 µl were required to achieve this degree of protection against serotype 3 and 4, respectively. These volumes of serum were then administered intraperitoneally 24 hours prior to IP challenge with 1000 CFU of serotype 3 (WU2) or serotype 4 (TIGR4) strain, respectively. Filtered culture supernatants of ΔCPS WU2 or TIGR4 strain were used as negative controls. In mice that were passively immunized with sera containing the relevant anti-capsule antibodies, the survival rate was <20% in the presence of as little as 0.2 µl of WU2 culture supernatant (containing about 0.03 µg type 3 CPS) whereas about 25 µl of TIGR4 culture supernatant (containing about 0.12 µg type 4 CPS) was required to abolish protection against the type 4 challenge (Fig 4).
DISCUSSION

Currently, a 0.35 µg/ml threshold of anti-capsular antibody for each serotype is used as a correlate of protection for licensure of pneumococcal conjugate vaccines. This correlate of protection has been used for the licensure of vaccines containing additional serotypes without the need for formal efficacy studies prior to regulatory approval. A recent study from the United Kingdom evaluated serotype-specific effectiveness and determined correlates of protection for each serotype included in PCV13. Serotype-specific correlates of protection varied widely, with a correlate of protection higher than 0.35 for several serotypes (11). In particular, for serotype 3, the calculated correlate of protection was 2.83 µg/ml, highest for all the serotypes represented in PCV13, raising of course the concern that thresholds for this and other serotypes may need to be revised.

Our study points to a possible mechanistic explanation for the finding that higher anti-capsular antibody concentrations may be required for protection against type 3. Indeed, our results demonstrated that compared to other serotypes included in PCV13, ST3 strain releases the most CPS during in vitro growth and infection in mice which then interferes with antibody-mediated killing in an opsonophagocytic assay and reduces the protective efficacy of pre-existing anti-CPS Ab in a murine model of sepsis. The extent to which the phenomenon of CPS release observed in vivo occurs in infection of humans remains to be determined, but is a plausible explanation for the observation that higher concentrations of antibodies may be required in infants and children to ensure protection against type 3 pneumococcal
disease. This hypothesis also is consistent with the finding that OPA titers that apparently suffice to confer protection against other pneumococcal serotypes are insufficient to ensure protection against serotype 3 (11).

PCVs prevent pneumococcal carriage as well as disease caused by the vaccine-containing serotypes. For example, immunization with 7-valent PCV (PCV7) resulted in marked decrease in the incidence of invasive diseases caused by the PCV7 vaccine serotypes (18). However, it is not yet clear to what extent type 3 conjugates are protective against disease or carriage caused by serotype 3. In studies from the US and the United Kingdom, protective effectiveness for the serotype 3 component of PCV13 was not significant (9, 11, 19). In fact, prior to the licensure of PCV13, studies of an experimental PCV11 vaccine that contained a conjugate of serotype 3 surprisingly showed no protection against AOM due to serotype 3 (19), which resulted in removal of that particular serotype from the vaccine.

With respect to carriage, a randomized trial evaluating the comparative efficacy between PCV13 and PCV7 failed to demonstrate reduction of acquisition of nasopharyngeal colonization by serotype 3 among PCV13 recipients, whereas there was a clear and significant reduction in cumulative acquisition of the serotypes that are included in PCV13 but not in PCV7 in recipients of PCV13 (with the exception of serotype 5, which was not prevalent enough for evaluation) (20). A more recent study from Massachusetts also has shown no change in serotype 3 carriage rate.
after PCV13 introduction, although the numbers of serotype 3 cases overall were
admittedly quite small (21).

Some of the studies suggested that low immunogenicity to serotype 3 conjugate may
be the underlying reason for breakthrough AOM or carriage (19, 20). While this is
certainly possible, the data presented in our study suggest that the failure of the
serotype 3 conjugate could also be due to CPS release by the organism, or put
differently, that the phenomenon of CPS3 release results in the higher protective
antibody threshold that is required. Inhibition of anti-CPS antibody by the released
serotype 3 CPS can be explained in two different ways. Released CPS can inhibit
anti-PCS antibody opsonisation by absorbing free unbound antibody. It is also
possible that CPS on which antibody was already bound can be released from the
bacteria. From our data, serotype 3 CPS concentration ratio of culture supernatant to
bacterial surface was greater than 1, suggesting higher antibody-absorbing capacity
by the CPS in culture supernatant.

It is important of course to acknowledge that the data presented here rely only on in
vitro and mouse in vivo studies. To what extent can the data shown in our studies
be extrapolated to the pathophysiological context in humans? Our results showed
that in mice, with a marginal protective anti-type 3 CPS antibody titer, the protection
can be completely abolished in the presence of as little as 0.03 μg of free type 3
CPS that could be released by approximately 10^4 CFU of WU2 strain in vivo (the
average CPS release post IP infection is about 30 μg/10^7 CFU as shown in Fig.2). In
contrast, for a ST4 strain, the abolishment of protection requires about 0.12 μg of free type 4 CPS which could not be reached even with $10^7$ CFU of ST4 strain in vivo (Fig.2) Although the density of bacteremia associated with significant disease in humans is not known, studies with *Haemophilus influenzae* type b revealed that 1000 bacteria/ml of blood (counted by direct plating on solidified media) was associated with meningitis (22).

In summary, our results suggest that the failure of the serotype 3 conjugate in PCV13 may be a direct consequence of the CPS release by the organism, a process that differentiates type 3 from other serotypes included in current conjugate vaccines. While higher antibody concentrations can overcome this phenomenon as shown here, alternative strategies (such as protein-based or whole cell vaccines, see review (23)) may be required for optimal protection against serotype 3 disease.
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Competing interests

Competing interests: FZ, YJL and RM have research funding related to vaccine research from National Institutes of Health, the Bill & Melinda Gates Foundation, PATH, Boston Children’s Hospital and Takeda Pharmaceutical Company. RM has received honoraria or consulting fees in the past from Merck and GSK. He is also a member of the scientific advisory boards of Genocea Biosciences, Arsanis Biosciences, and Advanced Inhalation Therapies. FZ, YJL and RM are scientific founders and consultants for, and own equity in, Affinivax, a biotechnology company focused on vaccine development. RM is also a member of the board of directors of Affinivax. EHC declares no conflicts of interest.

These data were presented in part at the 9th International Symposium on Pneumococci and Pneumococcal Diseases, Hyderabad, India, March 9-13, 2014.
Figure legends

1. Fig. 1. Comparison of surface CPS and CPS released in the culture supernatant by serotype and capsule synthesis pathway. Each symbol represents the mean measurement (3 to 4 repeat measurements per strain). Bars represent the median of various strains in the same serotype. Sup: culture supernatant. Surf: surface.

2. Fig. 2. CPS release in blood at 24 hours following intraperitoneal infection by various serotypes. A. CFU counts in sera after infection. Each symbol represents one mouse. (n=5 mice per group). Bars represent Median. B. CPS concentration in sera after infection (normalized to μg/10^7 CFU). Each symbol represents one mouse. Bars represent the median. Statistical analysis was performed using the Mann Whitney test.

3. Fig. 3. Inhibition of opsonophagocytic killing in the presence of released type3 CPS. A. OPKA titer of rabbit sera pre- and post-immunization with type 3 MAPS complex. B. Inhibition of opsonophagocytic killing against WU2 strain in the presence of different amounts of purified type3 CPS (diluted in the culture supernatant of ΔCPS WU2 strain) or wild type WU2 culture supernatant (containing 150 μg/mL of released type 3 CPS). The killing rate in the culture supernatant of ΔCPS WU2 strain without added CPS is defined as 100%. B. Inhibition of opsonophagocytic killing against WU2 strain in the presence of mouse sera post WU2 IP infection. The concentration of released type3 CPS in mouse sera was measured by inhibition ELISA. 30 μL of mouse sera was added to OPKA. The killing rate in the presence of naïve mouse
sera was defined as 100%. A standard inhibition curve was generated by adding different amounts of purified type3 CPS diluted in naïve mouse sera.

4. Fig 4. Inhibition of passive protection by adding culture supernatant of ST3 or ST4 pneumococcal strain. Mice (n=5 per group) received passive transfer with 40 µL or 20 µL of anti-ST3 or anti-ST4 rabbit sera, respectively, one day before infection. Mice were then infected by intraperitoneal injection with 1000 CFU of WU2 or TIGR4 resuspended in the culture supernatant of the corresponding ΔCPS strain with the addition of different amounts of WU2 or TIGR4 culture supernatant. WU2 culture supernatant contains 150 µg/mL of released type 3 CPS; TIGR4 culture supernatant contains 4.7 µg/mL of released type 4 CPS. There was no detectable CPS in the culture supernatant of ΔCPS strains. Mice were monitored for 7 days after infection. A and B, Survival curves after WU2 (A) or TIGR4 (B) IP infection. C, Summary of the inhibitory impact of WU2 or TIGR4 culture supernatant on passive protection. Statistical analysis was done using Log-rank (Mantel-Cox) test, comparing each group to the animals that received only supernatant from the related ΔCPS strain. n.s., not significant.
Table 1. Pneumococcal strains used in the current work

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Strains were obtained from the ABCs collection of the CDC (CDC/ABC); Dr. David Briles (DEB), Kate O'Brien (KOB) or a collection of clinical isolates in the Malley laboratory (RM).
REFERENCES


Fig. 1

CPS (g/10^7 CFU)
Fig. 3

A. 

B. 

C. 

% Survival

% Inhibition of bacterial killing

% Inhibition of bacterial killing

Added PS (ng)

Mouse sera post WU2 infection

Purified type 3 CPS + naive mouse sera

WU2 + Purified type 3 CPS

WU2 + Culture sup

Added PS (ng)

Dilutions
Fig. 4