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A Semi-Synthetic Glycoconjugate Vaccine Candidate for Carbapenem-Resistant Klebsiella pneumoniae

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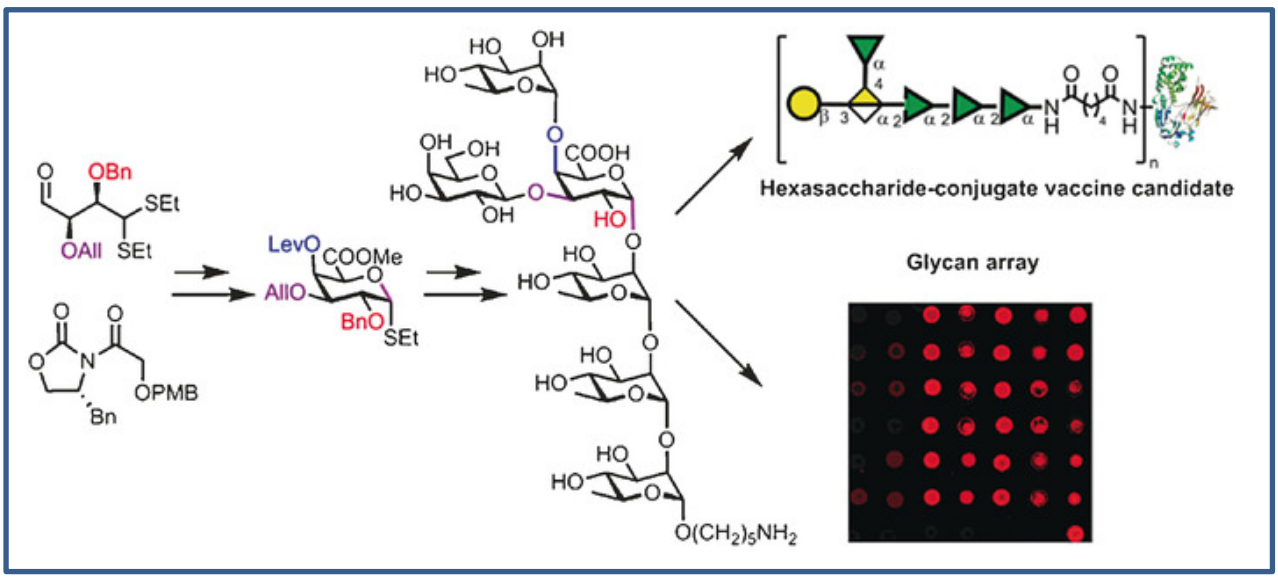

Resisting the Resistant: A hexasaccharide lead antigen has been identified en route to developing a vaccine against carbapenem-resistant *Klebsiella pneumoniae* (CR-*K*p), a deadly agent in hospital-acquired infections.

A semi-synthetic glycoconjugate vaccine candidate for carbapenem-resistant *Klebsiella pneumoniae*

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Abstract:

Hospital acquired infections are an increasingly serious health concern. Infections caused by carpabenem-resistant *Klebsiella pneumoniae* (CR-*Kp*) are especially problematic, with a 50% average survival rate. CR-*Kp* are isolated from patients with ever greater frequency: 7% within the EU but 62% in Greece. At a time when antibiotics are becoming less effective, no vaccines to protect from this severe bacterial infection exist. Here, we describe the convergent [3+3] synthesis of the hexasaccharide repeating unit from its capsular polysaccharide and related sequences. Immunization with the synthetic hexasaccharide **1** glycoconjugate resulted in high titers of cross-reactive antibodies against CR-*Kp* CPS in mice and rabbits. Whole cell ELISA was used to establish the surface staining of CR-*Kp* strains. The antibodies raised were found to promote phagocytosis. Thus, this semi-synthetic glycoconjugate is a lead for the development of a vaccine against a rapidly progressing, deadly bacterium.

Klebsiella pneumoniae (*Kp*) is the leading cause of nosocomial respiratory and urinary tract infections as well as bacteremia, primarily among newborns and immunocompromised patients.[1] Carbapenem-resistant *Kp* (CR-*Kp*) are now commonly encountered in hospitals worldwide. Outbreaks occur with increasing frequency,[2] and these strains are highly contagious[3] and cause high morbidity and mortality.[4] Isolates belonging to the sequence type 258 (ST258) that expresses *Kp* carbapenemase are largely responsible for the global spread of CR-*Kp*.[5] Efficacious vaccination of risk groups is direly needed as treatment options for CR-*Kp* are diminishing.

Successful vaccines against streptococci[6] and meningococci[7] are based on conjugates of capsular polysaccharides (CPS) with carrier proteins. Currently, no vaccines for prophylactic or therapeutic use against *Kp* are available. These Gram-negative pathogens are surrounded by CPS and lipopolysaccharides (LPS). Active immunization with LPS-containing vaccines holds severe risks associated with endotoxic components such as Lipid A. [8] CPS, on the other hand, are highly immunogenic and nontoxic. [9] A 24-valent *Klebsiella* vaccine manufactured by the Swiss Serum and Vaccine Institute, composed of CPS antigens from *Kp* and *K. oxytoca* that are expressed by about 70% of *Klebsiella* strains associated with bacteremia, proved safe and immunogenic in Phase I clinical trials.[10] Challenges associated with glycan isolation rendered the vaccine too costly and development ended following Phase I clinical trials.[11]

The production of well-defined glycans by chemical synthesis is attractive as it is reliable and scalable method. Long lasting, T-cell dependent protection from bacterial infections can be provided by oligosaccharide antigens that are linked to a carrier protein[12] as demonstrated by a semi-synthetic glycoconjugate vaccine against *Haemophilus influenzae* type b marketed in Cuba.[13] Semi-synthetic oligosaccharide vaccine candidates against *Shigella*[14], meningococci[15] and streptococci[16]are currently under preclinical evaluation.

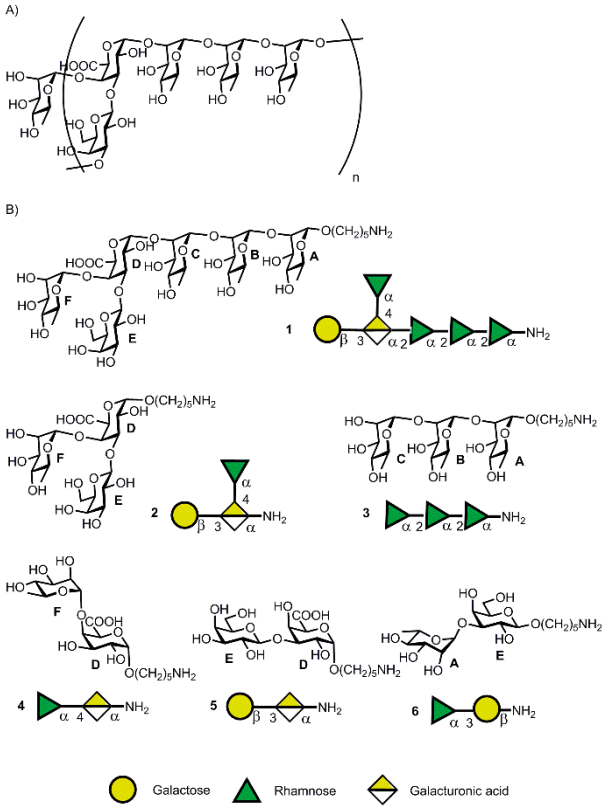

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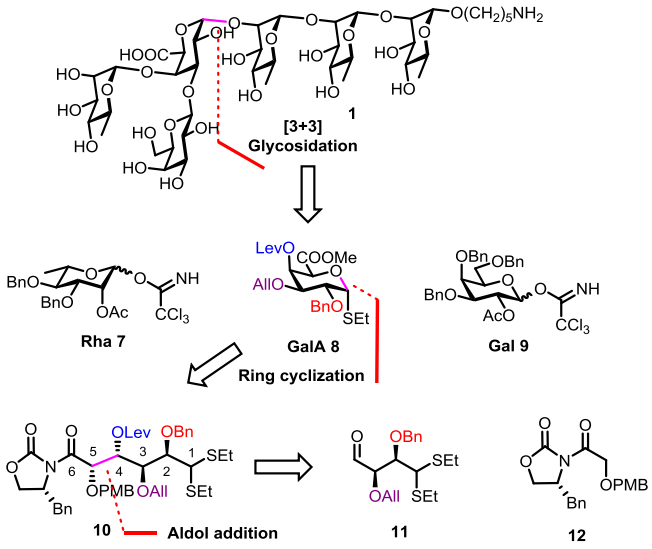
Figure 1. A) Hexasaccharide RU from two CR-*Kp* clones isolated during a 2011 hospital outbreak; B) Synthetic hexasaccharide **1** and substructures **2**-**6** used for immunological studies.

Clinical CR-*Kp* isolates responsible for a serious 2011 outbreak killing six of the 18 infected patients were found to have identical CPS comprising a hexasaccharide repeating unit (RU) (Figure 1 a).[17] This RU resembles CPS of other isolated serotypes even though it is not identical. A vaccine based on hexasaccharide antigen **1** may provide a much needed defense against these devastating pathogens (Figure 1 b).

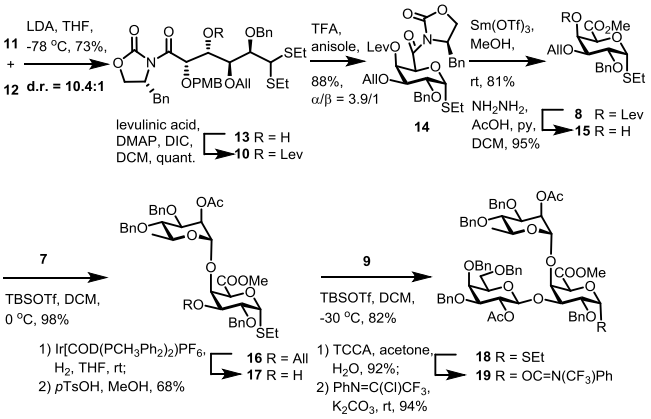
Herein, we report the synthesis of hexasaccharide **1** as well as related substructures **2**-**6** (Figure 1 b) and their immunological evaluation *in vivo*. Glycan microarray studies revealed that only hexasaccharide **1** is recognized by monoclonal antibody (MAb) 1C9 that cross-reacts with CR-*Kp* CPS[20]. The glycoconjugate hexasaccharide CRM197-**1** vaccine candidate is immunogenic in mice, and is promising for further preclinical development to protect against CR-*Kp* outbreaks.



Scheme 1. Retrosynthetic analysis of hexasaccharide **1**.

To prepare the glycans, a convergent [3+3] approach based on three monosaccharide building blocks, Rha **7**[18], GalA **8**, and Gal **9**[19], was selected (Scheme 1). Differentially protected GalA **8** carrying benzyl ether (Bn), allyl ether (All) and levulinyl ester (Lev) groups at C2, C3 and C4 was to be synthesized by cyclization of thioacetal **10**, which is derived from aldehyde **11** and auxiliary **12** via aldol addition. For glycan immobilization on array surfaces and subsequent immunization studies an aminopentanol linker is included at the reducing end. The synthesis of differentially protected GalA building block **8** via a *de novo* approach [21-23] commenced with treatment of auxiliary **12** with LDA at -78 °C, followed by addition of aldehyde **11** (Supporting Information) to furnish aldol adduct **13** (d.r. = 10.4:1; Scheme 2).[24] Levulination afforded fully protected thioacetal **10**; treatment with trifluoroacetic acid and anisole led to cleavage of *p*-methoxybenzyl ether and concomitant cyclization produced the desired thioglycoside **14** as a mixture of anomers (α/β = 3.9/1). Isolation of the α-product followed by methanolysis of the chiral auxiliary under mild acidic conditions provided GalA **8.**[25]

The assembly of trisaccharide donor **18** commenced by selective removal of the C4-Lev ester of **8** upon exposure to hydrazine, providing alcohol **15** (Scheme 2). Glycosylation of **15** with rhamnosyl building block **7** afforded disaccharide **16** as the α-anomer. A two step removal of the allyl group via the enol ether provided disaccharide **17**.[26] Glycosylation of **17** with galactosyl trichloroacetimidate **9** proceeded smoothly at -30°C to give trisaccharide thioglycoside **18** as a single β-anomer, which was converted to glycosyl *N*-phenyltrifluoroacetimidate **19**[27].

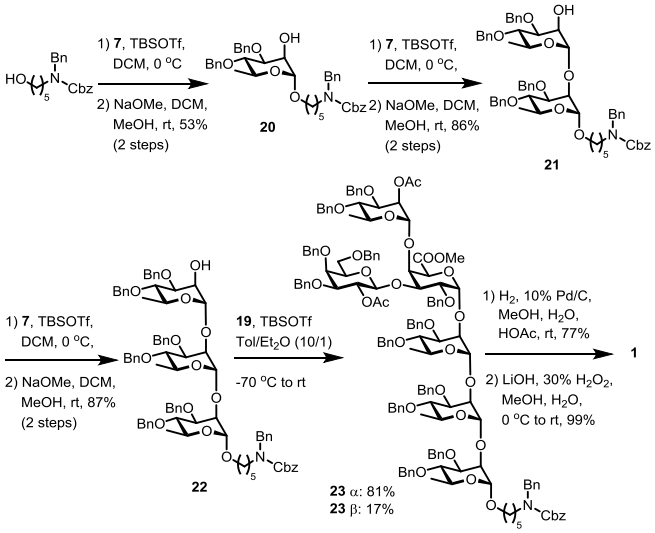


Scheme 2. Synthesis of trisaccharide building blocks **18** and **19**.

An iterative glycosylation and deprotection strategy was employed to construct trisaccharide acceptor **22**, using rhamnosyl trichloroacetimidate building block **7** (Scheme 3). Coupling of Rha **7** with the protected aminopentanol linker afforded α-linked rhamnoside; subsequent removal of the C2-acetate by treatment with sodium methoxide provided acceptor **20**. Repeating this glycosylation-deprotection sequence twice with Rha **7** produced trisaccharide acceptor **22**.

With donors **18** and **19** as well as acceptor **22** in hand, the key [3+3] glycosylation was investigated. Identification of effective reaction conditions proved challenging due to the poor reactivity and sterics of the uronic acid donor **18** and the inert C-2 hydroxyl of the trisaccharide acceptor **22**.[28-29] Ultimately, thioglycoside **18** failed to react with acceptor **22**. However, *N*-phenyltrifluoroacetimidate **19**, after some optimization, reacted smoothly in a mixture of toluene and diethyl ether at -70 °C to provide the hexasaccharide **23** (Scheme 3). The desired configuration of the α-isomer of **23** was confirmed by the coupling constant between galacturonic acid *C*-1 and *H*-1 ($^1J_{C\text{-}H}$ = 172 Hz; Supporting Information).[30]

The initial plan for the global deprotection of **23,** relying on the initial cleavage of acetate and methyl esters followed by hydrogenation of the benzyl groups, did not meet with success as only the pentasaccharide β-elimination product was obtained (data not shown). In contrast, when hydrogenolysis was carried out first, followed by saponification, the target hexasaccharide **1** was obtained in 76 % yield (Scheme 3). NMR spectra of synthetic hexasaccharide **1** were in good agreement with those reported for the CPS from the CR-*Kp* outbreak[17] (Supporting Information). The substructures **2**-**6** (Figure 1 b) were also synthesized for epitope mapping studies (Supporting Information).



Scheme 3. [3+3] Glycosylation to complete hexasaccharide **1**.

The synthetic glycans along with the native CR-*Kp* CPS were printed on microarray slides and incubated with the partially protective mouse IgM monoclonal antibody (MAb) 1C9 raised against CR-*Kp* CPS [20]. Mab 1C9 specifically recognizes RU hexasaccharide **1** as no binding was observed for the other synthetic glycans (Figure 2 A). Immunogenic structures that induce cross-reactive immune responses to native CPS can be determined by CPS-based inhibition studies[15]. Accordingly, the MAb 1C9 was first incubated with CR-*Kp* CPS and *S. pneumoniae* type 3 (ST3) CPS was used as a negative control (Figure 2 C). MAb binding was abolished after pre-incubation with CR-*Kp* CPS but not with other synthetic glycans (Figures 2 B and 2 E), suggesting that hexasaccharide **1** effectively mimics the natural CPS epitope. Therefore, **1** was further immunologically characterized.

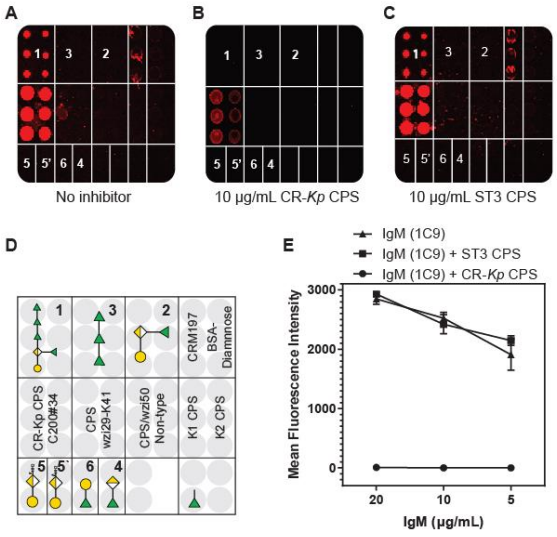


Figure 2. CR-*Kp* CPS inhibits binding of MAb 1C9 to hexasaccharide **1**. (A) Glycan microarray was incubated with different amounts of MAb 1C9. Bound antibodies were detected using goat anti-mouse IgM Alexa-flour 594 secondary antibodies. (B) Pre-adsorption of MAb 1C9 with CR-Kp CPS (10 µg/mL) followed by incubation with the printed array inhibits binding to **1**. (C) ST3 CPS was used as negative control. (D) Glycan microarray printing pattern. (E) Mean Fluorescence Intensities (MFI) measured for the inhibition assay. Data are represented as mean ± SD (triplicate) following subtraction of background values.

Carbohydrate based, type 2 T-independent antigens are poorly immunogenic [31] and small synthetic glycans alone are ineffective in eliciting an immune response. Attachment of synthetic glycan antigens to carrier proteins that induce a T cell response enhances glycan immunogenicity and triggers specific memory responses. We used CRM197, a nontoxic mutant of diphtheria toxin, as a carrier because it is a component of several marketed conjugate vaccines.[32] The *p*-nitrophenyladipate ester[33] derivative of hexasaccharide **1** was covalently coupled to CRM197 to yield the CRM197-**1** conjugate with an average of 7.5 hexasaccharide **1** molecules per CRM197 (Figure S2).

The immunogenicity of the CRM197-**1** conjugate formulated either with Freund's adjuvant (FA) or human approved aluminum hydroxide was assessed. Mice were immunized subcutaneously and received two booster shots, one on day 14 and one on day 35 (Figure 3 A). High antibody titers were detected using glycan microarrays one week after the final immunization (Figures 3 B and 3 C). The antibody response was adjuvant dependent as conjugate formulated with FA induced more cross-reactive antibodies than the alum formulation. Antibodies against FA formulated conjugate recognize both trisaccharides **2** and **3** while those elicited in response to the alum formulation cross-react only with trisaccharide **3** (Figures 3 B and 3 C). Antibody binding to hexasaccharide **1** and trisaccharide **3** was similar for both formulations (Figure 3 C). Interestingly, the FA adjuvanted conjugate also induces CPS cross-reactive antibodies while the alum formulation does not (Figure 3 D). Synthetic glycan antigens elicited a significantly stronger antibody response in rabbits when compared to mice.[34] Rabbits were immunized with CRM197-**1** conjugate formulated with alum, an adjuvant approved for use in humans.[35] The serological analysis using glycan microarrays and ELISA revealed that CRM197-**1** conjugate elicits a robust immune responses against native CR-*Kp* CPS (Figure S3). Collectively, these results demonstrate that the CRM197-**1** conjugate is immunogenic in mice and rabbits and produces antibodies that are cross-reactive with native CR-*Kp* CPS.

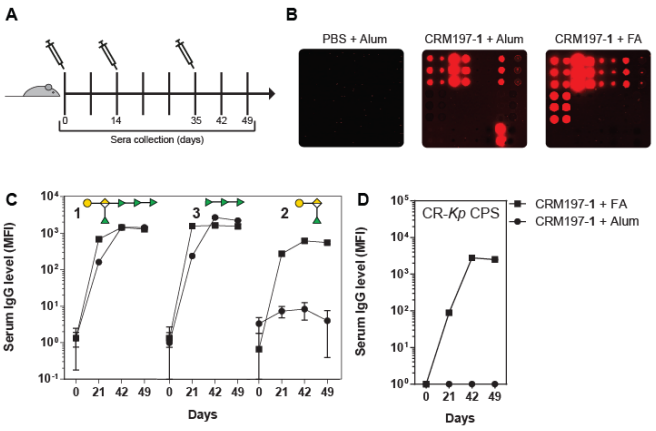


Figure 3. Mice immunized with CRM197-**1** produce serum antibodies that recognize CPS. (A) Schematic immunization schedule. Mice (three individuals per group) were immunized on day 0 with CRM197-**1** conjugate (3 µg sugar per dose) formulated in aluminum hydroxide (alum) or FA and boosted on days 14 and 35 with the same amount of antigen subcutaneously in 100 µL volume. Control mice received PBS with alum. (B) A representative microarray scan following incubation of pooled sera from each group. (C and D) The MFI of serum antibody cross-reactivity with synthetic structures and native CPS were expressed as mean ± SD of three microarray spots.

In order for CRM197-**1** to qualify as a vaccine lead, immunological memory and anti-CPS antibody mediated protection through challenge has to be demonstrated. CR-*Kp*

strains are avirulent in mice; therefore, there is currently no established animal challenge model available to test CRM197-**1**[20]. The production of different types of antibodies is being used to assess the immunological response *in vivo*. The majority of antibodies produced by mice in response to immunization with CRM197-**1** are IgG1 as determined by glycan array analyses (Figures 4 A and 4 C) while less IgG2a and IgG3 are observed (Figures 4 D and 4 E). The CRM197-**1** specific total serum antibody level (IgG and IgM) is significantly higher than the PBS group (Figures 4 F and 4 G). The strong immunological response and antibody class switch underscores the potential of hexasaccharide **1** as a vaccine lead against CR-*Kp*.

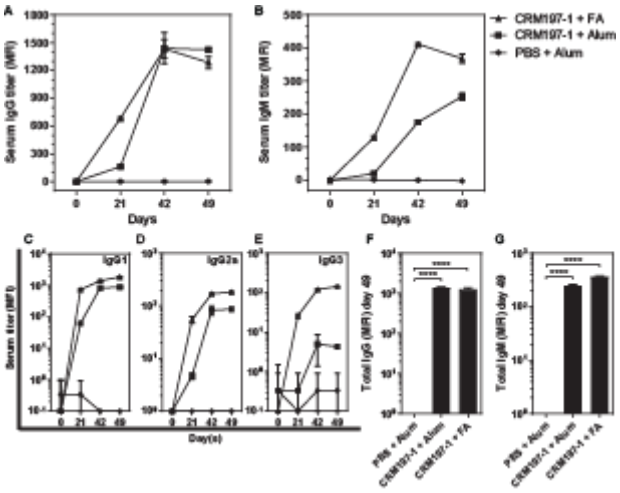


Figure 4. Hexasaccharide **1** specific serum antibody isotyping. Immunization with CRM197-**1** conjugate induces high antibody titers in mice. Mouse sera obtained at different time points were qualitatively analyzed using glycan microarrays for multiple antibody isotypes. The MFI of hexasaccharide **1** specific serum (A) IgG, (B) IgM antibodies at different time points. The IgG isotype antibodies as expressed in MFI values of (n=3) are shown in (C) IgG1, (D) IgG2a, and (E) IgG3, respectively. Total serum antibody titers at day 49 (F) IgG and (G) IgM. MFIs for antibody titers was plotted as mean ± SD (triplicate) and analyzed by unpaired t-test using GraphPadPrism. *p* values of < 0.05 were considered statistically significant. **P*≤ 0.1, ***P*≤ 0.05, ****P*≤ 0.01 and *****P*≤ 0.001.

In order for an antibody to be protective *in vivo*, it has to be able to bind to *Klebsiella* and induce antibody dependent *in vitro* phagocytosis that typically correlates with *in vivo* protection.[36] Therefore, we assessed the surface binding of CRM197-**1** specific antibodies to intact *Klebsiella* strains (both *carbapenem-resistant* and sensitive). The whole cell ELISA assay suggests that anti-CRM197-**1** antibodies bind broadly to CR-*Kp* strains while there was no binding observed to other capsular antigen expressing stains (Figure 5 A).

The functional relevance of the antibodies induced in response to immunization with CRM197-**1** conjugate was assessed by a opsonophagocytic killing assay (OPKA). Differentiated HL-60 cells were incubated with CR-*Kp* strains 85/12 and NRZ-00319 preopsonized with anti-CRM197-**1** antibodies. The phagocytic activity of *Klebsiella* was assessed by plating on blood agar plates and was expressed as percent killing. CRM197-**1** conjugate immunization elicits opsonic antibodies that exhibit phagocytic activity against CR-*Kp* strains tested (Figures 5 B-E). Antibodies promoting bacterial killing 50 % or more were considered biologically significant. The antibody titer values for 50 % killing for 85/12 and NRZ-00319 are 153.6 and 134.2, respectively (Figure 5 C and 5 E). CRM197-**1** conjugate produce functional antibodies that promote uptake and killing of *Klebsiella* by phagocytic cells in mice as well as in rabbits (Figure S4).

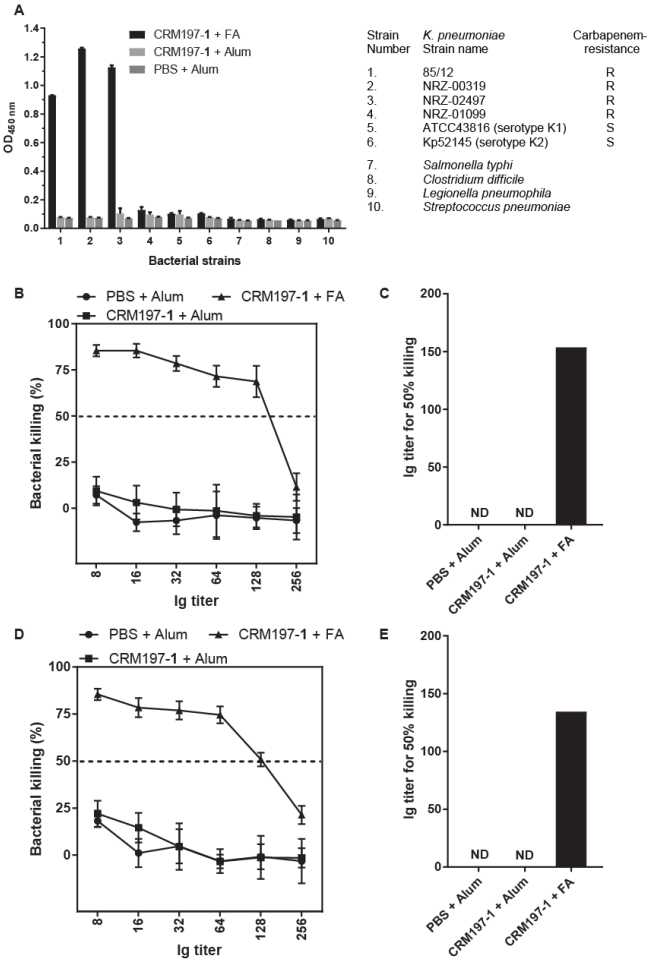


Figure 5. (A) Surface binding of CRM197-**1** antibodies was analyzed by whole cell ELISA. Antibody binding was assessed for *CR-Kp* strains 85/12, NRZ-00319, NRZ-02497, NRZ-01099, carbapenem sensitive strains ATCC43816 (serotype K1), Kp52145 (serotype 2) and other capsular antigen expressing strains *Salmonella typhi*, *Legionella pneumophila, Clostridium difficile and Streptococcus pneumoniae*. Surface staining was performed with murine anti-CRM197-**1** antibodies (n=3) raised with alum and FA formulation. PBS with alum was served as control. Anti-CRM197-**1** antibodies promote the phagocytosis of CR-*Kp*. The differentiated HL-60 cells were incubated with 85/12 (B and C) and NRZ-00319 (D and E) strains pretreated with anti-CRM197-**1** antibodies and control sera, and *Klebsiella* survival was assessed after 90 min. Percent killing of *Klebsiella* were calculated based on viable *Klebsiella* colonies obtained relative to no sera control. Values represent an independent experiment done in duplicates. Values represent mean ± SD. ND, not detected.

In summary, synthetic oligosaccharides help to identify and define glycan cell-surface epitopes as a basis for vaccine development against bacterial infections. The synthesis of CR-*Kp* CPS hexasaccharide RU **1** and related glycans **2-6** enabled glycan microarray analyses that revealed that hexasaccharide **1** is selectively recognized by the monoclonal antibody 1C9, which cross-reacts with CR-*Kp* CPS.[17] The CRM197-**1** conjugate elicits high antibody titers in mice and rabbits and shows immunological memory in mice, cross-reacting with native CPS. Immunization with CRM197-**1** conjugate elicits CR-*Kp* CPS specific antibodies that bind the surface of intact bacteria and result *in vitro* phagocytic killing of CR-*Kp Klebsiella* strains. The full potential of CRM197-**1** as a vaccine candidate can be evaluated only once a suitable animal challenge model, currently being established for CR-*Kp* strains, is available.

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Keywords:*Klebsiella pneumoniae*•antibiotic resistance•hexasaccharide total synthesis•vaccines

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