



# Antibody-Mediated Killing of Carbapenem-Resistant ST258 *Klebsiella pneumoniae* by Human Neutrophils

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**ABSTRACT** Carbapenem-resistant *Klebsiella pneumoniae* is a problem worldwide. A carbapenem-resistant *K. pneumoniae* lineage classified as multilocus sequence type 258 (ST258) is prominent in the health care setting in many regions of the world, including the United States. ST258 strains can be resistant to virtually all clinically useful antibiotics; treatment of infections caused by these organisms is difficult, and mortality is high. As a step toward promoting development of new therapeutics for ST258 infections, we tested the ability of rabbit antibodies specific for ST258 capsule polysaccharide to enhance human serum bactericidal activity and promote phagocytosis and killing of these bacteria by human neutrophils. We first demonstrated that an isogenic *wzy* deletion strain is significantly more susceptible to killing by human heparinized blood, serum, and neutrophils than a wild-type ST258 strain. Consistent with the importance of capsule as an immune evasion molecule, rabbit immune serum and purified IgG specific for ST258 capsule polysaccharide type 2 (CPS2) enhanced killing by human blood and serum *in vitro*. Moreover, antibodies specific for CPS2 promoted phagocytosis and killing of ST258 by human neutrophils. Collectively, our findings suggest that ST258 CPS2 is a viable target for immunoprophylactic and/or therapeutics.

**IMPORTANCE** Infections caused by carbapenem-resistant *K. pneumoniae* are difficult to treat, and mortality is high. New prophylactic approaches and/or therapeutic measures are needed to prevent or treat infections caused by these multidrug-resistant bacteria. A strain of carbapenem-resistant *K. pneumoniae*, classified by multilocus sequence typing as ST258, is present in many regions of the world and is the most prominent carbapenem-resistant *K. pneumoniae* lineage in the United States. Here we show that rabbit antibodies specific for capsule polysaccharide of ST258 significantly enhance human serum bactericidal activity and promote phagocytosis and killing of this pathogen by human neutrophils. These studies have provided strong support for the idea that development of an immunotherapy (vaccine) for carbapenem-resistant *K. pneumoniae* infections is feasible and has merit.

**KEYWORDS** *Klebsiella*, antibiotic resistance, antibody, carbapenems, neutrophils, vaccines

*Klebsiella pneumoniae* is a commensal bacterium of the intestine, but it can cause infections in individuals with significant comorbidities and/or risk factors, such as major surgery or immunosuppression. Infections caused by *K. pneumoniae* are primarily hospital-associated infections. Magill et al. reported that *K. pneumoniae* and *K. oxytoca* together caused ~10% of all infections among 183 United States hospitals tested (1). These infections included respiratory tract infections (pneumonia), surgical site infections, urinary tract infections, and bloodstream infections (1). The problem of the high

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burden of infections caused by *Klebsiella* spp. is compounded by antibiotic resistance. Although *K. pneumoniae* is known historically for its resistance to  $\beta$ -lactam antibiotics, the worldwide emergence of carbapenem-resistant *K. pneumoniae* strains that are susceptible only to colistin, tigecycline, and/or gentamicin is a major concern (2, 3). In addition, some strains of carbapenem-resistant *K. pneumoniae* are resistant to all clinically relevant antibiotics and treatment of infections caused by such organisms is difficult (4). Mortality associated with infections caused by carbapenem-resistant *K. pneumoniae* is relatively high (e.g., ~30% to ~48% in selected studies of bloodstream infections) (5–7), and new approaches for prophylaxis or treatment are needed.

A carbapenem-resistant *K. pneumoniae* strain classified by multilocus sequence typing as sequence type 258 (ST258) remains the most prominent lineage in United States hospitals (8–10). Carbapenem resistance in ST258 is conferred by *K. pneumoniae* carbapenemase (KPC), which is encoded by *bla*<sub>KPC</sub> within a transposon (Tn4401) that is present on a plasmid (11). Recent genome analyses indicate that the two major clades of ST258 encode different capsule polysaccharide (CPS) types by gene clusters known as *cps-1* and *cps-2* (12–14). These two capsule types remain predominant among ST258 clinical isolates worldwide, and *cps-2* isolates are more abundant than *cps-1* isolates in many geographic regions (10, 13, 15–17). The contribution of CPS to the success of ST258 outside antibiotic resistance remains incompletely determined.

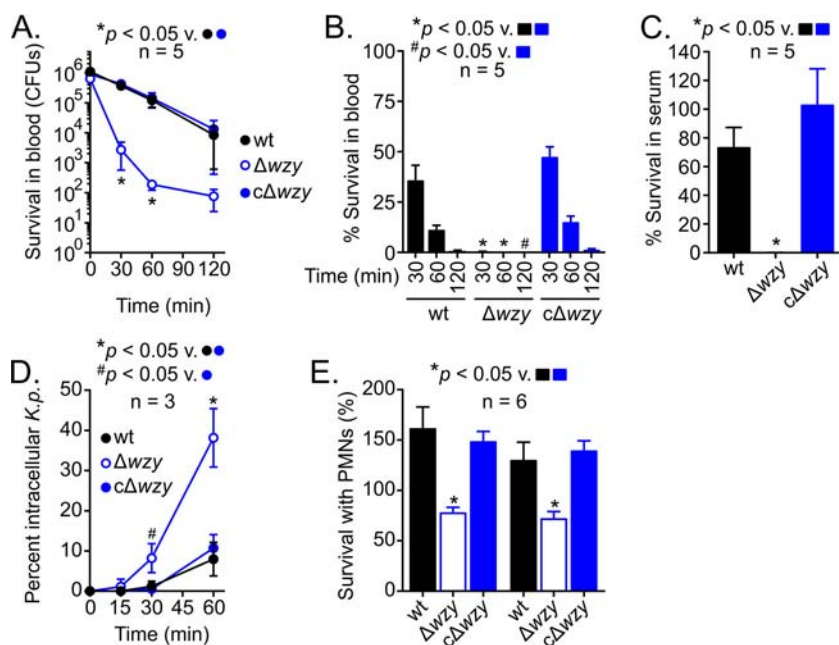
We demonstrated previously that *cps-1* or *cps-2* ST258 clinical isolates are largely resistant to phagocytosis by human neutrophils but that any ingested bacteria are killed readily (18). Moreover, optimal killing of ST258 by serum complement occurs in the presence of naturally occurring immunoglobulin G (IgG) (19). In aggregate, those and other previous studies provided support to the idea that immunotherapy (a vaccine approach) can be considered for prevention/treatment of infections caused by carbapenem-resistant *K. pneumoniae* (20). As a first step toward testing the validity of an immunotherapy approach, we generated antibodies specific for CPS1 and CPS2 of ST258 clinical isolates and tested their ability to enhance serum bactericidal activity and promote phagocytosis and killing by human neutrophils.

## RESULTS AND DISCUSSION

**ST258 CPS contributes to evasion of innate host defense.** CPS is known to contribute to *K. pneumoniae* virulence, a characteristic attributed largely to resistance to complement-mediated killing and phagocytosis (21–24). The roles played by *K. pneumoniae* CPS are varied and strain specific (23, 25, 26). A gene known as *wzy* is required for capsule biosynthesis in many species of bacteria, including *K. pneumoniae* (27–29). Previous studies performed with serotype K1 *K. pneumoniae* strains demonstrated that a *wzy*-like gene known as *magA* confers resistance to serum complement and phagocytosis (28, 30, 31). As a first step toward determining whether CPS has contributed to the success of ST258 as a human pathogen, we evaluated the survival rates of wild-type and isogenic *cps-2* mutant ( $\Delta wzy$ ) ST258 strains in human blood and serum *in vitro*. Compared to that seen with the wild-type ST258 strain, the survival of the  $\Delta wzy$  ST258 strain was reduced significantly in human blood and serum (e.g., the survival rates in 100% serum were 73.7%  $\pm$  13.6% for the wild-type strain and 0.1%  $\pm$  0.03% for the mutant strain;  $P < 0.05$ ) (Fig. 1A to C). Survival of the  $\Delta wzy$  strain in heparinized blood and serum was restored fully by complementation with *wzy* expressed from a plasmid (Fig. 1A to C).

We next compared levels of phagocytosis and killing of wild-type and  $\Delta wzy$  ST258 strains by human neutrophils (Fig. 1D and E). Consistent with the ability of CPS to inhibit complement function, the  $\Delta wzy$  ST258 strain was ingested and killed by neutrophils to a significantly greater extent than the wild-type or complemented mutant strains (Fig. 1D and E). Taken together, these results indicate that the ST258 CPS contributes to evasion of innate host defenses and thereby promotes survival in humans.

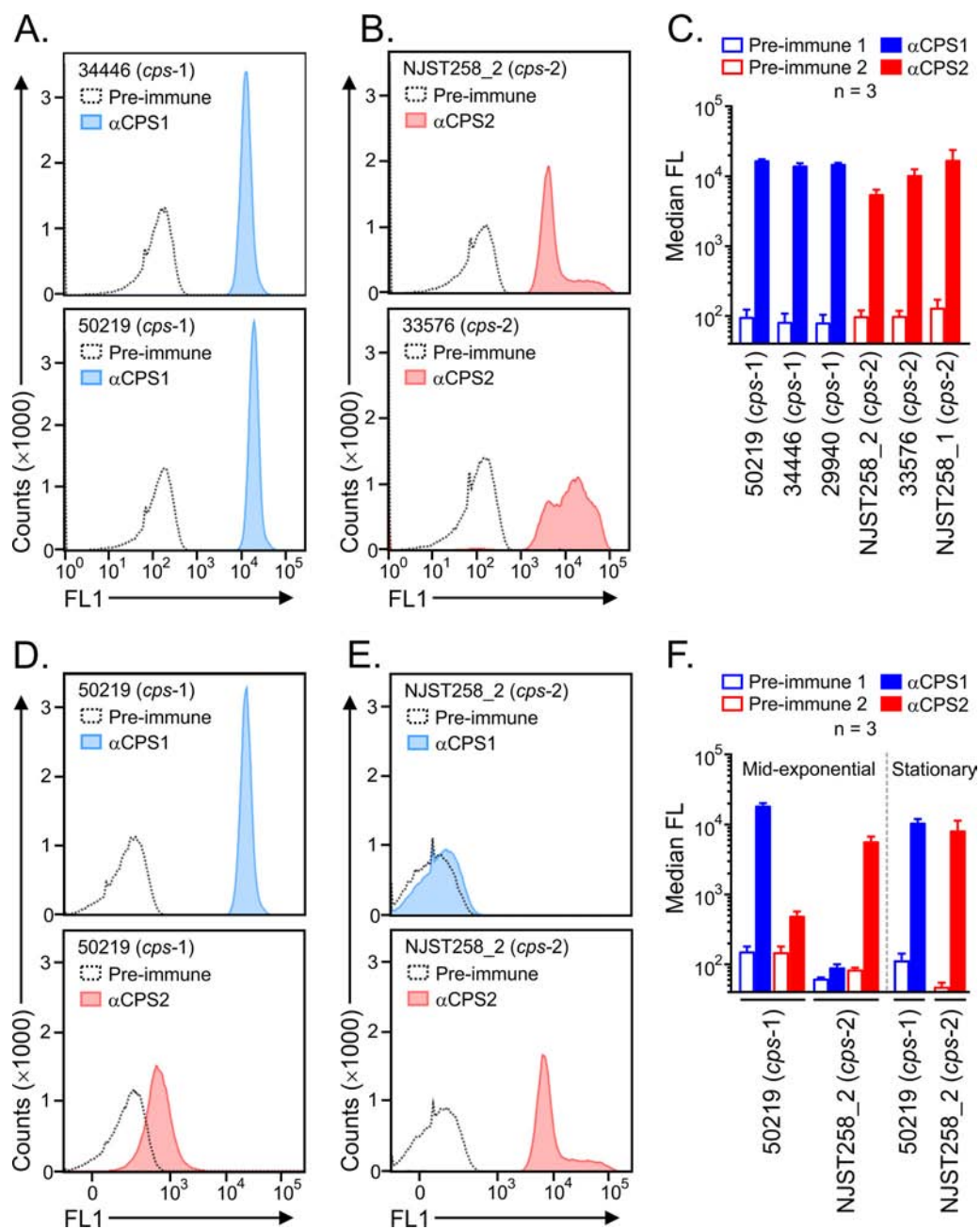
**Generation of capsule-specific rabbit antibodies.** Inasmuch as the majority of carbapenem-resistant ST258 *K. pneumoniae* clinical isolates in the United States can be



**FIG 1** CPS contributes to evasion of killing by normal human serum and neutrophils. (A to C) Differential survival of ST258 (*cps-2*) wild-type, isogenic  $\Delta wzy$  mutant ( $\Delta wzy$ ), and complemented  $\Delta wzy$  mutant ( $c\Delta wzy$ ) strains in heparinized human blood (A and B) and 100% normal human serum (C) quantified in CFUs (A) and percent survival (B and C). (D) Phagocytosis of wild-type,  $\Delta wzy$ , and  $c\Delta wzy$  strains by human neutrophils was measured as described in Materials and Methods. *K.p.*, *K. pneumoniae*. (E) Analysis of rates of survival of the indicated strains during a phagocytic interaction with human neutrophils was performed using a ratio of  $\sim 10$  CFU per neutrophil. Results are presented as means  $\pm$  standard errors of the means (SEM) for the indicated number (*n*) of experiments. Data were analyzed using repeated-measures one-way ANOVA and Tukey's posttest.

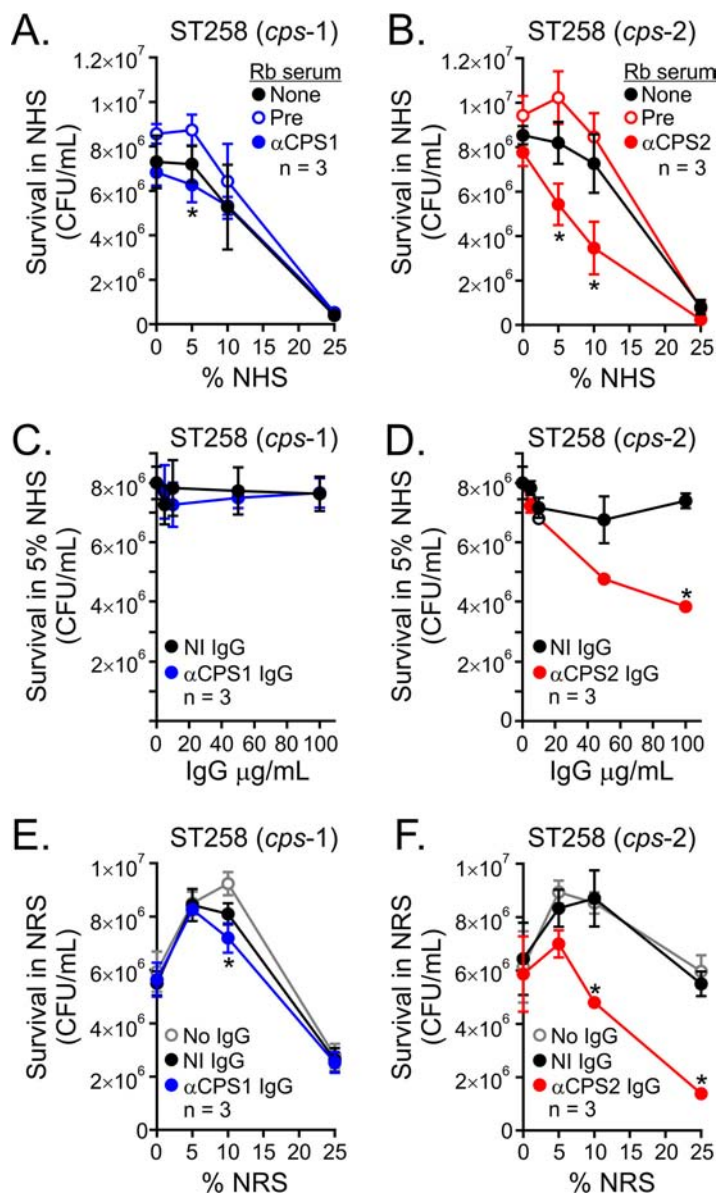
classified as *cps-1* or *cps-2* (13), we used purified CPS1 and CPS2 from representative ST258 clinical isolates to generate rabbit polyclonal antiserum specific for *cps-1* and/or *cps-2* strains (Fig. 2). CPS preparations were highly immunogenic, and antiserum from rabbits immunized with either CPS1 (anti-CPS1) or CPS2 (anti-CPS2) contained antibodies that bound the surface of multiple *cps-1* or *cps-2* isolates (Fig. 2A to C). Although there was recognition (albeit limited) of *cps-1* isolate 50219 with anti-CPS2 (Fig. 2D; compare upper and lower panels), anti-CPS1 failed to bind NJST258\_2, a representative *cps-2* isolate (Fig. 2E). These results were verified in preliminary flow cytometry assays performed with 2 additional *cps-1* isolates (34440 and 29940) and *cps-2* isolates (NJST258\_1 and 33576) and are likely explained by differences noted in the compositions of CPS1 and CPS2 (see Table S1 in the supplemental material).

**CPS antibodies promote serum bactericidal activity.** We demonstrated previously that *cps-1* and *cps-2* ST258 clinical isolates have generally comparable survival rates in normal human serum (NHS) (19). That said, a subset of those isolates (3 of 20), including NJST258\_1 (*cps-2*), were killed in 5% NHS (19). Depletion of IgG from human serum increased survival of this isolate, providing support to the idea that naturally occurring antibodies promote complement-mediated killing of ST258 (19). Indeed, serum from healthy blood donors contained IgG and/or IgM that bound to the surface of *cps-1* or *cps-2* isolates (see Fig. S1 in the supplemental material). To determine whether anti-CPS1 and anti-CPS2 contain antibodies that promote complement-mediated killing of ST258, we tested the ability of 1% anti-CPS1 or anti-CPS2 to augment bactericidal activity of NHS (Fig. 3). Consistent with the ability of IgG to promote deposition of complement and formation of the membrane attack complex, serum bactericidal activity was enhanced significantly by anti-CPS2 in the presence of 5% and/or 10% NHS (Fig. 3A and B). The ability of anti-CPS1 to augment bactericidal activity of NHS was limited by comparison (compare panels A and B in Fig. 3). This



**FIG 2** Generation of rabbit antiserum specific for CPS1 and CPS2. (A and B) ST258 clinical isolates were stained with anti-CPS1 (A), anti-CPS2 (B), or preimmune serum (A and B) as indicated and evaluated by flow cytometry. Data shown are from a representative experiment performed at least three times. (C) Quantitation of the median fluorescence (FL) of three selected *cps-1* or *cps-2* ST258 isolates. Results are presented as means  $\pm$  SEM from three separate flow cytometry experiments. (D) *cps-1* isolate 50219 stained with anti-CPS1 (top) or anti-CPS2 (bottom). (E) *cps-2* isolate NJST258\_1 stained with anti-CPS1 (top) or anti-CPS2 (bottom). Data shown in panels D and E are from representative experiments. (F) Quantitation of data shown in panels D and E. Results are presented as means  $\pm$  SEM from three separate flow cytometry experiments.

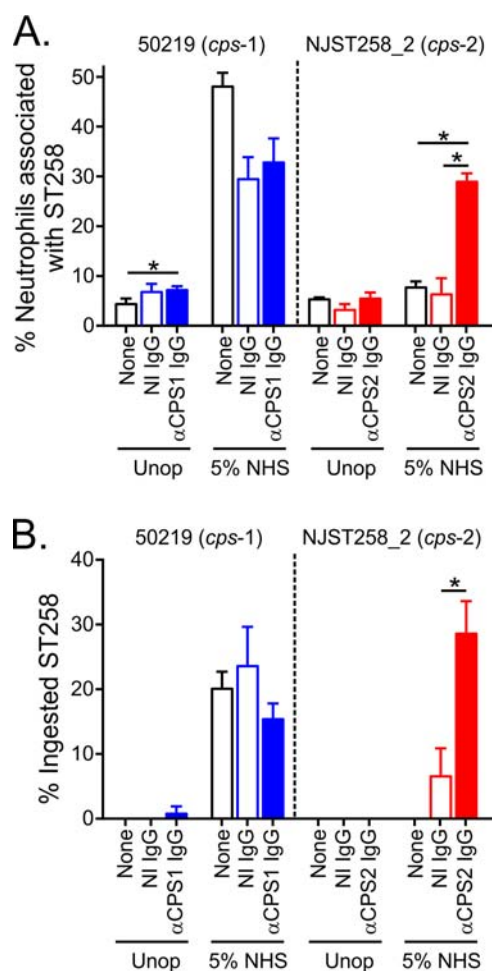
finding was surprising to us, since the titer of anti-CPS1 (~64,000), which was evaluated by flow cytometry with live bacteria, was greater than that of anti-CPS2 (~16,000). In addition, this *cps-1* isolate (50219) is killed at significant levels in 25% NHS and is therefore not fully resistant to complement-mediated bactericidal activity (Fig. 3A). The findings determined with immune serum were recapitulated by using IgG purified from anti-CPS1 or anti-CPS2 (Fig. 3C and D). The limited ability of anti-CPS1 and IgG anti-CPS1 to augment the bactericidal activity of NHS was not likely related to the



**FIG 3** CPS-specific antibody enhances serum bactericidal activity. (A and B) ST258 (*cps-1*) isolate 50219 (A) or ST258 (*cps-2*) isolate NJST258\_2 (B) was incubated with 1% anti-CPS1, 1% anti-CPS2, or 1% preimmune serum (Pre) in NHS as indicated, and survival was measured as described in Materials and Methods. Rb serum, rabbit serum. (C to F) Isolate 50219 (C and E) or isolate NJST258\_2 (D and F) was incubated with the indicated concentration of purified anti-CPS1 IgG, anti-CPS2 IgG, or nonimmune (NI) IgG mixed with 5% NHS (C and D) or 5% normal rabbit serum (NRS) (E and F), and survival was measured as described in Materials and Methods. Results are presented as means  $\pm$  SEM from three separate experiments. Data in panels A and B were analyzed using repeated-measures one-way ANOVA and Tukey's posttest. \*,  $P < 0.05$  (versus samples with rabbit preimmune serum and no rabbit serum). Data in panels C to F were analyzed with a two-tailed Student  $t$  test. \*,  $P < 0.05$  (for samples containing anti-CPS IgG versus those with NI IgG).

presence of (or to competition with) naturally occurring antibodies in NHS or unique to isolate 50219, since the results were similar with normal rabbit serum (Fig. 3E and F) or with another *cps-1* isolate (34446; survival was  $100.7\% \pm 14.4\%$  versus  $104.0\% \pm 30.2\%$  in 5% NHS containing  $100 \mu\text{g/ml}$  nonimmune [NI] IgG versus  $100 \mu\text{g/ml}$  anti-CPS1 IgG;  $n = 3$ ). Taken together, these findings demonstrate that antibody specific for CPS2 enhances serum bactericidal activity.

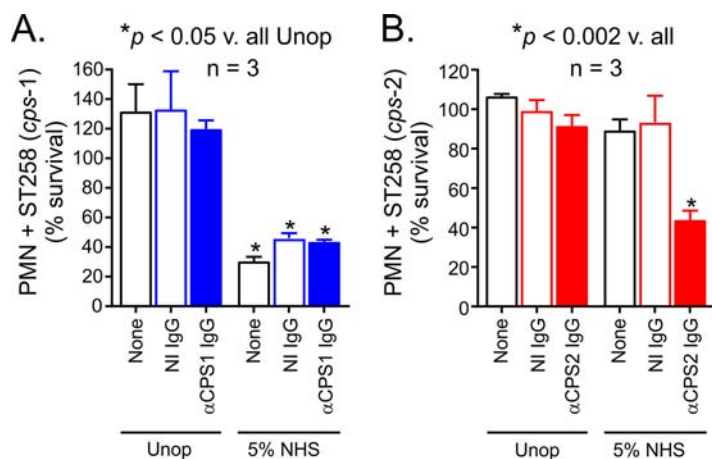
**Capsule-specific antibody promotes phagocytosis and killing of ST258 by human neutrophils.** We next tested the ability of IgG purified from anti-CPS1 or



**FIG 4** CPS-specific antibody promotes binding and phagocytosis of ST258 by human neutrophils. (A) Association of ST258 isolate 50219 (*cps-1*) or isolate NJST258\_2 (*cps-2*) with human neutrophils with or without 5% NHS in the absence (None) or presence of 100  $\mu$ g/ml anti-CPS1 IgG, anti-CPS2 IgG, or nonimmune (NI) IgG was determined as described in Materials and Methods. Data are expressed as the percentages of neutrophils that had associated bacteria, which includes surface-bound and ingested bacteria. (B) Phagocytosis of ST258 isolates by human neutrophils was determined using fluorescence microscopy as described in Materials and Methods. Phagocytosis data are expressed as the percentages of associated bacteria (bound plus ingested) that were intracellular. The assays described in the legends for panels A and B were performed at a ratio of 1 CFU per neutrophil, and results are presented as means  $\pm$  SEM from three separate experiments. \*,  $P < 0.05$  (as determined with repeated-measures one-way ANOVA and Tukey's posttest). Unop, unopsonized (no serum added).

anti-CPS2 to enhance binding and phagocytosis of ST258 by human neutrophils. Although we reported previously that there was limited neutrophil phagocytosis of *cps-1* and *cps-2* isolates (18), isolate 50219 (*cps-1*) was not among those tested. Unexpectedly, 50219 was bound and ingested by human neutrophils cultured in NHS alone, and the levels of these processes were not increased by addition of anti-CPS1 IgG (Fig. 4). In contrast, significantly more neutrophils were associated with NJST258\_2 (*cps-2*) following addition of anti-CPS2 IgG than were seen in control assays lacking CPS-specific antibody (e.g., 29.2%  $\pm$  1.4% of neutrophils in assays containing anti-CPS2 IgG had associated bacteria, compared with 6.6%  $\pm$  3.0% in assays containing NI IgG;  $P = 0.003$ ) (Fig. 4A). In accordance with these data, anti-CPS2 IgG increased phagocytosis (ingestion) of NJST258\_2 significantly (Fig. 4B).

We previously reported that any ST258 isolates ingested by human neutrophils were readily destroyed, albeit phagocytosis was limited under those assay conditions (18). To determine whether killing of our representative *cps-1* and *cps-2* ST258 isolates by neutrophils would be reflected by the phagocytosis results (Fig. 4), we evaluated



**FIG 5** CPS2-specific IgG promotes killing of ST258 by human neutrophils. ST258 isolate 50219 (*cps-1*) (A) or isolate NJST258\_2 (*cps-2*) (B) was cultured with human neutrophils with or without 5% NHS in the absence (None) or presence of 100  $\mu$ g/ml anti-CPS1 IgG, anti-CPS2 IgG, or nonimmune (NI) IgG as indicated. Assays were performed at a ratio of 1 CFU per neutrophil. Results are presented as means  $\pm$  SEM from three separate experiments. \*,  $P < 0.05$  (as determined with repeated-measures one-way ANOVA and Tukey's posttest). Unop, unopsonized (no serum added).

bacterial survival during phagocytic interactions with human neutrophils with or without CPS-specific antibody (Fig. 5; see also Fig. S2). Consistent with the ability of NHS alone to promote phagocytosis of the ST258 *cps-1* isolate, bacterial survival was reduced significantly by the activity of human neutrophils in the presence of NHS (survival was 30.4%  $\pm$  3.0% for assays that included the use of NHS versus 131.7%  $\pm$  18.3% for assays lacking NHS) (Fig. 5A). This neutrophil bactericidal activity was not augmented by addition of anti-CPS1 IgG (Fig. 5A), a finding that was not surprising to us given the relatively high level of bacterial killing by neutrophils under these assay conditions. It is possible that the naturally occurring CPS1-specific antibodies present in the NHS from our pool of healthy blood donors were sufficient to promote optimal uptake of the *cps-1* ST258 isolate tested here (Fig. S1). Such a hypothesis is in general consistent with a similar phenomenon known to occur for *Staphylococcus aureus* (i.e., NHS contains naturally occurring *S. aureus* antibodies and in turn promotes phagocytosis), another human commensal organism and opportunistic pathogen. In contrast to the results determined with the *cps-1* ST258 isolate, the presence of NHS alone failed to promote significant killing of the *cps-2* isolate (NJST258\_2) by human neutrophils (Fig. 5B). Most notably, survival of NJST258\_2 was reduced significantly by addition of anti-CPS2 IgG to neutrophil assays containing NHS (survival was 93.4%  $\pm$  13.5% for assays containing NI IgG compared with 43.3%  $\pm$  4.8% for those with anti-CPS2 IgG;  $P = 0.0007$ ) (Fig. 5B). These results demonstrate that CPS-specific antibodies promote phagocytosis and killing of carbapenem-resistant ST258 *K. pneumoniae*.

**Concluding remarks.** Infections caused by antibiotic-resistant bacteria represent a major problem globally. There is continued emergence and reemergence of antibiotic-resistant bacteria, including carbapenem-resistant *K. pneumoniae*. For example, ceftazidime-avibactam was shown recently to be effective for treatment of carbapenem-resistant *K. pneumoniae* (32, 33), but, as with many antibiotics, resistance can develop during treatment (34). Therefore, new preventive or therapeutic approaches are needed. Immunoprophylaxis and immunotherapy approaches based on the use of anti-CPS antibodies or hyperimmune intravenous immunoglobulin (IVIG) from patients immunized with *Klebsiella* CPS were developed in the 1980s and 1990s (35–38). Importantly, such approaches circumvent the problem of antibiotic resistance. The anti-CPS vaccine approached worked well to protect rodents from death in experimental models of severe *K. pneumoniae* infection (39, 40). The vaccine was evaluated for safety in humans (35), and the use of CPS-specific hyperimmune IVIG decreased the incidence and severity of *Klebsiella* infections in a human clinical trial

(41). Previous work also demonstrated that vaccines (either monovalent or polyvalent) can protect against multiple (as many as 71) *Klebsiella* capsule types (35, 42). The majority of ST258 clinical isolates in the United States and many other regions of the world are composed of one of only two capsule polysaccharide types (*cps*-1 or *cps*-2) (10, 13, 15, 17). Therefore, as a starting point, a vaccine that targets the most prominent strains of carbapenem-resistant *K. pneumoniae* need be effective only against *cps*-1 and *cps*-2 strains. The number of *cps* types to be included in a potential vaccine could then be expanded based on clinical and molecular epidemiology. Here we show a proof of concept for an immunotherapy approach that could be developed for prevention or treatment of carbapenem-resistant ST258 *K. pneumoniae* infections. It is noteworthy that our results were obtained using serum and neutrophils obtained from healthy individuals, who are not susceptible to ST258 infections in general. Thus, the effective ability of anti-CPS antibody to promote killing of ST258 might be increased in the susceptible host (e.g., an individual with comorbidities or immunosuppression). An immunotherapy approach for *K. pneumoniae* could be extended to target lineages other than ST258, and such work is ongoing (43, 44). Moreover, recent studies indicated that antibodies specific for *K. pneumoniae* lipopolysaccharide work synergistically with antibiotics to improve outcomes in mouse infection models (20). Such an approach could be adapted for use with antibodies specific for CPS or with a combination of antibodies specific for CPS and lipopolysaccharide. It will be important in future studies to test the ability of this immunotherapy approach to protect against severe disease or death in animal infection models.

## MATERIALS AND METHODS

**Bacterial strains and culture.** *Klebsiella pneumoniae* isolates NJST258\_1 (*cps*-2), NJST258\_2 (*cps*-2), 33576/1793 (*cps*-2), 34446/1805 (*cps*-1), 29940/1775 (*cps*-1), and 50219/1787 (*cps*-1) were reported and/or characterized previously (12, 18, 19). Three of these strains/clinical isolates (NJST258\_1, NJST258\_2, and 34446) were tested previously in a serum bactericidal activity assay. We demonstrated previously that NJST258\_1 is serum sensitive (survival is ~25% in 5% NHS) relative to NJST258\_2 and 34446, which survive fully under the same assay conditions. After considering serum sensitivity and resistance, we selected isolates 50219 and NJST258\_2 for generation of rabbit antiserum because these isolates are representative of ST258 clades 1 and 2, which constitute the majority of carbapenem-resistant *Klebsiella pneumoniae* isolates from selected health care facilities in the United States (13). Isolate 33576 is a carbapenem-susceptible ST258 clinical isolate (*cps*-2) that was used to generate the isogenic capsule polysaccharide mutant ( $\Delta$ wzy) and complemented mutant ( $\Delta$ wzy) strains (see below for details). Bacteria from frozen stocks were inoculated into Luria-Bertani (LB) broth and cultured overnight with shaking at 37°C. Cultures were diluted 1:200 into fresh media the following day and cultured to the desired phase of growth.

A Red/ET recombination system was used to generate an ST258 isogenic *wzy* deletion strain (the  $\Delta$ wzy mutant) according to the manufacturer's protocol (Quick and Easy *Escherichia coli* gene deletion kit; Gene Bridges, Heidelberg, Germany), but with slight modification. Briefly, a linear DNA fragment, i.e., an FRT-PGK-gb2-arr3-FRT cassette (rifampin resistance gene cassette) with 50-bp arms homologous to DNA upstream and downstream of *wzy*, was amplified by PCR and used to replace *wzy* in *K. pneumoniae* isolate 33576. Positive transformants were confirmed by using gene-specific primers. Complementation of the  $\Delta$ wzy strain was conducted by cloning *wzy* from the wild-type strain into a pGlow vector (Invitrogen), followed by electroporation into the  $\Delta$ wzy strain.

**Ethics statement and isolation of human neutrophils.** Venous blood or heparinized venous blood was obtained from healthy volunteers in accordance with a protocol approved by the Institutional Review Board for Human Subjects at the National Institute of Allergy and Infectious Diseases (protocol 01-I-N055). All volunteers gave informed consent prior to participation in the study.

Human neutrophils were isolated from heparinized human blood using a standard method, which includes dextran sedimentation followed by Hypaque-Ficoll gradient centrifugation as previously described (45, 46). Granulocytes comprised  $99.1\% \pm 0.3\%$  of the leukocytes in the neutrophil preparations (sampled over a 1-month period), and viability was  $>99\%$  as assessed by flow cytometry (FACSCelesta; BD Biosciences) as reported previously (45).

**Purification and analysis of capsule polysaccharide.** *K. pneumoniae* capsule polysaccharide was extracted and purified using two separate published methods with some modifications. For immunization of rabbits, CPS was extracted essentially as reported by Cunha et al. (47) prior to purification by gel filtration chromatography. In brief, bacteria were cultured overnight in LB, often in 250-ml cultures, and then pelleted by centrifugation at  $17,000 \times g$  for 30 min at 4°C. The pellet was resuspended in extraction buffer (0.1% Zwittergent 3-14–50-mM sodium citrate buffer, pH 4.5) at 1/10 original culture volume and heated at 42°C in a water bath for 30 min. Bacteria were centrifuged again as described above, and the CPS-containing supernatant was aspirated and filter sterilized. The crude extraction material was purified further with a HiLoad 16/600 Superdex 200 gel filtration column (GE Healthcare Life Sciences, Pittsburgh, PA). Fractions containing CPS peaks (as determined by a standard uronic acid assay) were pooled, and



the buffer was changed to 0.9% injection-grade saline solution by using a Centricon Plus-70 filter unit (EMD Millipore) (molecular weight cutoff [MWCO], 100,000). The amount of endotoxin in each CPS preparation was determined with a kit assay (QCL-1000 end point chromogenic LAL assay) as described by the manufacturer (Lonza Inc.).

For analysis of CPS composition, CPS was extracted from culture supernatants using the method of Cryz et al. (48). Bacteria were cultured for 16 h in 250 ml HYEM medium (2% [wt/vol] Hy-Case SF, 0.3% [wt/vol] yeast extract, 2% [wt/vol] maltose) at 37°C with shaking and then pelleted by centrifugation at  $8,000 \times g$  for 30 min. Supernatant was aspirated and filtered using a 0.22- $\mu\text{m}$ -pore-size polyethersulfone (PES) filter. CPS was precipitated with N-cetyl-N,N,N-trimethylammonium bromide (CETAB) (0.5% final concentration) at room temperature (RT) for 30 min with stirring. Samples were centrifuged at  $4,200 \times g$  for 30 min, supernatant was aspirated, and CPS was dissolved in 50 ml 1 M  $\text{CaCl}_2$ . CPS was precipitated by addition of 200 ml (80% [vol/vol]) ethanol. Samples were centrifuged again at  $4,200 \times g$  for 30 min, and CPS pellets were dissolved in 25 to 50 ml  $\text{H}_2\text{O}$ . CPS was concentrated as needed by using a Centricon Plus-70 filter unit and stored at 4°C until used. CPS preparations were then analyzed by gas chromatography and mass spectrometry, size exclusion chromatography, and nuclear magnetic resonance (NMR) spectroscopy at the Complex Carbohydrate Research Center, University of Georgia, Atlanta, GA (see Table S1 in the supplemental material).

**Production of rabbit antibody.** The animal protocol used for these studies was reviewed and approved by the Institutional Animal Care and Use Committee, Rocky Mountain Laboratories, NIAID/NIH (protocol RML 2017-004). In brief, New Zealand white rabbits (2 to 4 kg each) were inoculated with up to 500  $\mu\text{g}$  purified *K. pneumoniae* CPS–0.5-ml pharmaceutical grade saline solution mixed 1:1 with TiterMax Gold adjuvant (Sigma-Aldrich). The CPS/TiterMax Gold inoculum was administered with a 23-gauge needle as follows: 0.05 ml was injected intramuscularly into each hind leg of each rabbit, and 0.1 ml was injected subcutaneously into 4 sites behind the shoulders and along the back. Each rabbit received an inoculum of 0.5 ml in total, which could include up to 10  $\mu\text{g}$  *K. pneumoniae* endotoxin (lipopolysaccharide) per rabbit. Rabbits were subjected to boosting without TiterMax Gold every 3 to 4 weeks using the same immunization procedure as that described above. Blood and serum were collected prior to initial immunization (preimmune serum) and after each boost. Nonimmune (NI) serum was also collected from healthy rabbits that were not immunized. Serum was prepared according to standard methods, and aliquots were frozen at  $-80^\circ\text{C}$  until use. Serum from rabbits immunized with CPS1 or CPS2 was labeled anti-CPS1 or anti-CPS2, respectively.

IgG was purified from anti-CPS1 or anti-CPS2 and NI serum using a protein G HP SpinTrap column according to the instructions of the manufacturer (GE Healthcare Life Sciences). Antibody was concentrated using an Amicon Ultra-15 centrifugal filter unit (Millipore Sigma, Burlington, MA) (10K MWCO) and suspended in sterile Dulbecco's phosphate-buffered saline (DPBS) to the desired volume, and IgG levels were measured using a Pierce bicinchoninic acid (BCA) protein assay kit (Thermo, Fisher Scientific, Waltham, MA).

**Detection of surfaced-expressed CPS with flow cytometry.** Bacteria were cultured to the mid-logarithmic or late stationary (overnight) phase of growth in LB broth. Aliquots (200  $\mu\text{l}$ ) of culture were pelleted by centrifugation ( $2,400 \times g$  for 4 min at RT), washed in DPBS, and suspended in 500  $\mu\text{l}$  DPBS containing 2% (wt/vol) bovine serum albumin (blocking buffer) on ice for 60 min. Bacteria were pelleted again by centrifugation and then resuspended in 500  $\mu\text{l}$  DPBS. Aliquots (100  $\mu\text{l}$ ) of bacteria were combined with 100  $\mu\text{l}$  of prediluted (1:1,000) anti-CPS1, anti-CPS2, or preimmune serum (1:2,000 final dilution) and incubated on ice for 30 min. Alternatively, bacteria were incubated on ice for 30 min with IgG (5  $\mu\text{g}/\text{ml}$ ) purified from anti-CPS1, anti-CPS2, or NI serum. Samples were diluted with 800  $\mu\text{l}$  wash buffer (0.8% bovine serum albumin [BSA]–DBPS), and bacteria were pelleted as described above. Bacteria were resuspended in 100  $\mu\text{l}$  of prediluted (1:500 in DBPS) secondary antibody, i.e., AffiniPure F(Ab)<sub>2</sub> fragment goat anti-rabbit IgG (H+L) conjugated to fluorescein isothiocyanate (FITC) (Jackson ImmunoResearch, West Grove, PA), and incubated on ice for 30 min. At the end of the incubation period, 800  $\mu\text{l}$  of wash buffer was added to each tube, and bacteria were pelleted by centrifugation and resuspended in 200- $\mu\text{l}$  wash buffer. Bacteria were analyzed by flow cytometry (FACSCelesta flow cytometer; BD Biosciences).

Antibody titers were determined using live bacteria combined with flow cytometry. The titers of anti-CPS1 and anti-CPS2 were determined according to the greatest dilution at which there was still a difference in surface binding compared to rabbit NI serum (negative control; 1:2,000). The presence of CPS-specific antibody in NHS was measured as described above, except AffiniPure F(Ab)<sub>2</sub> fragment goat anti-human IgG (H+L) or goat anti-human IgG plus IgM (H+L) conjugated with FITC (Jackson ImmunoResearch) was used as the secondary antibody.

**Serum bactericidal activity.** Survival of bacteria in normal human serum (NHS) was determined using a published method (19). Assays for serum bactericidal activity were performed using NHS that was either prepared fresh or frozen once and thawed for use. Survival of the wild-type,  $\Delta\text{wzy}$ , and  $\text{c}\Delta\text{wzy}$  ST258 strains was determined in 100% NHS for 30 min at 37°C. The ability of anti-CPS1 or anti-CPS2 (diluted 1:100 in NHS; 1% final concentration) or of anti-CPS IgG to augment human serum bactericidal activity was determined using 5% to 25% NHS as indicated. Bacteria ( $\sim 2 \times 10^6$  CFU/ml) were combined with NHS and either 1% rabbit preimmune or immune serum or with anti-CPS IgG in RPMI medium to reach a final volume of 0.6 ml. Assay tubes were rotated gently for 1 h at 37°C. Aliquots from each assay tube were plated on LB, and colonies were enumerated the following day.

**Neutrophil phagocytosis and bactericidal activity.** Human neutrophil phagocytosis and bactericidal activity were determined using a published method (18), but with modifications.

Two separate assays were used to measure phagocytosis and killing of bacteria by human neutrophils. We used a published synchronized phagocytosis assay to determine the levels of phagocytosis and killing of wild-type and  $\Delta wzy$  and  $c\Delta wzy$  ST258 strains (18). Bacteria, used at a ratio of ~10 CFU per neutrophil, were not opsonized with NHS for these assays, because the  $\Delta wzy$  mutant would be killed by serum complement and this would confound interpretation of the results. We used a synchronized phagocytosis assay for these experiments because phagocytosis is optimal under these conditions (i.e., neutrophils are semiaherent and primed, and bacteria are in close proximity or in contact).

To measure the ability of antibody to promote polymorphonuclear leukocyte (PMN) bactericidal activity, we used a suspension phagocytosis assay. In brief, bacteria were left unopsonized or were preincubated with 5% NHS and 1% rabbit preimmune or immune serum or 5% NHS and 100  $\mu\text{g/ml}$  anti-CPS IgG or NI IgG for 10 min at room temperature in RPMI 1640 medium, HEPES (RPMI/H). Human PMNs were added to the assay mixtures at a 1:1 CFU-to-PMN ratio, and samples (0.6 ml final volume) were rotated gently for 1 h at 37°C. At the designated time, 0.1% saponin was added to the assay mixtures, which were then chilled on ice for 15 min to permeabilize and lyse PMNs. Aliquots of assay mixtures were plated on LB to determine CFU data. Percent survival was determined with the following equation: percent survival =  $\text{CFU}_{+PMN}/\text{CFU}_{-PMN} \times 100$ .

**Statistics.** Statistical analysis was performed with GraphPad Prism version 7.03 (GraphPad Software, Inc.). To determine data for comparisons of 3 or more samples, data were analyzed using one-way analysis of variance (ANOVA) or repeated-measures one-way ANOVA and Dunnett's or Tukey's posttest as indicated. Comparisons of 2 samples were made using a two-tailed Student's *t* test.

## SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mBio.00297-18>.

**FIG S1**, TIF file, 2.2 MB.

**FIG S2**, TIF file, 0.7 MB.

**TABLE S1**, DOCX file, 0.01 MB.

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S.D.K., B.N.K., and F.R.D. conceived and designed the study. B.N.K., R.P., and L.C. provided all bacterial strains and clinical isolates. A.R.P., B.F., and S.D.K. performed the experiments. A.R.P., S.D.K., and F.R.D. performed all of the data analyses and verification of data analyses. F.R.D. drafted the manuscript text and assembled the figures. The manuscript text was edited by S.D.K., A.R.P., and B.N.K., and all of us reviewed the final text.

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