The Diversity of Lipopolysaccharide (O) and Capsular Polysaccharide (K) Antigens of Invasive Klebsiella pneumoniae in a Multi-Country Collection

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Klebsiella pneumoniae is a common cause of sepsis and is particularly associated with healthcare-associated infections. New strategies are needed to prevent or treat infections due to the emergence of multi-drug resistant K. pneumoniae. The goal of this study was to determine the diversity and distribution of O (lipopolysaccharide) and K (capsular polysaccharide) antigens on a large (>500) global collection of K. pneumoniae strains isolated from blood to inform vaccine development efforts. A total of 645


**INTRODUCTION**

*Klebsiella pneumoniae* is a common Gram-negative bacterial cause of sepsis that contributes to high mortality and morbidity in hospitalized, immunosuppressed, and chronically ill patients. *Klebsiella* spp. were the third most common cause of healthcare-associated infections (9.9%) in acute-care hospitals in the United States in 2011 after *Clostridioides difficile* (12.1%) and *Staphylococcus aureus* (10.7%) (Magill et al., 2014). A follow-up study was performed in 2015 and showed that the prevalence of healthcare-associated infections was decreased compared to 2011. Further, *Klebsiella* spp. were responsible for ~5% of these infections with *C. difficile* (15%), *S. aureus* (11%) and *Escherichia coli* (10%) being the most common (Magill et al., 2018). Similarly, *K. pneumoniae* was responsible for 12% of healthcare-associated pneumonia infections in a point prevalence survey in acute care hospitals in the European Union in 2011–2012 (Walter et al., 2018). Many additional studies have highlighted the importance of *K. pneumoniae* as a cause of healthcare-associated infections (Meatherall et al., 2009; Folgori et al., 2016; Cai et al., 2017; Baykara et al., 2018).

The occurrence of multidrug-resistant (MDR) *K. pneumoniae* has been considered as an urgent threat worldwide (Lee et al., 2017). Limited therapeutic options for infection with MDR *K. pneumoniae* results in increased mortality, longer hospital stays and inflated healthcare costs (Cerceo et al., 2016). Given the emergence of multidrug resistant isolates, prevention of *K. pneumoniae* infections has become of paramount importance. Several groups have developed vaccines to prevent *K. pneumoniae* infection and these target capsular polysaccharide, extracellular vesicles and outer membrane proteins (Cryz et al., 1986a; Edelman et al., 1994; Chhibber et al., 2005; Lundberg et al., 2013; Lee et al., 2015). Likewise, we are developing a quadrivalent conjugate vaccine that targets the O1, O2, O3, and O5 polysaccharides of *K. pneumoniae* (Hegerle et al., 2018). We and others have shown that antibodies directed against the O polysaccharide are protective against wild-type *K. pneumoniae* in lethal infection models (Clements et al., 2008; Hegerle et al., 2018).

*Klebsiella pneumoniae* capsular polysaccharide (K antigen) and lipopolysaccharide (O antigen) are important virulence factors that can activate the innate immune system (Simoons-Smit et al., 1986; Shankar-Sinha et al., 2004). Additionally, these two antigens are used to differentiate *K. pneumoniae* isolates. More than 77 K antigens have been defined to date (Follador et al., 2016). Trautmann et al. (1997) determined that over 80% of *K. pneumoniae* isolates from Denmark belong to O1, O2, O3, and O5. This finding is corroborated by Follador et al. (2016) who sequenced more than 500 human and environmental isolates from United States, United Kingdom, Australia, Singapore, Indonesia, Laos, Nepal and Vietnam. Of these, only 134 out of the 500 isolates were from humans; 47 strains were from urine, 67 strains were isolated from blood, and 20 strains were from sputum (Follador et al., 2016). Nine serotypes have been proposed but in different combinations by separate groups O1, O2ab (O2a), O2a/O9 (O2aeh), O3, O4, O5, O7, O11, and O12 was proposed by Trautmann et al. (1997) whereas O1, O2, O2ac, O3, O4, O5, O7, O8, and O12 was proposed by Hansen et al. (1999). The O8 polysaccharide is similar to the O1 antigen and O9 is a subtype of the O2 antigen (Ørskov and Ørskov, 1984; Kelly et al., 1993). Serotyping O2 isolates is particularly complex due to the various O2 subtypes (O2a, O2ac, O2aeh, and O2afg) (Clarke et al., 2018). LPS O antigens are composed of D-galactans in O1 and O2 and mannans in O3 and O5 strains. Among galactans,

*K. pneumoniae* isolates were collected from the blood of patients in 13 countries during 2005–2017. Antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method. O antigen types including the presence of modified O galactan types were determined by PCR. K types were determined by multiplex PCR and *wzi* capsular typing. Sequence types of isolates were determined by multilocus sequence typing (MLST) targeting seven housekeeping genes. Among 591 isolates tested for antimicrobial resistance, we observed that 19.3% of isolates were non-susceptible to carbapenems and 62.1% of isolates were multidrug resistant (from as low as 16% in Sweden to 94% in Pakistan). Among 645 isolates, four serotypes, O1, O2, O3, and O5, accounted for 90.1% of *K. pneumoniae* strains. Serotype O1 was associated with multidrug resistance. Fifty percent of 199 tested O1 and O2 strains were *gmlABC*-positive, indicating the presence of the modified polysaccharide subunit D-galactan III. The most common K type was K2 by both multiplex PCR and *wzi* capsular typing. Of 39 strains tested by MLST, 36 strains were assigned to 26 known sequence types of which ST14, ST25, and ST258 were the most common. Given the limited number of O antigen types, diverse K antigen types and the high multidrug resistance, we believe that an O antigen-based vaccine would offer an excellent prophylactic strategy to prevent *K. pneumoniae* invasive infection.

**Keywords:** *Klebsiella pneumoniae*, multidrug resistance, vaccine, O antigen, K antigen
D-galactan I was found in O1 (with D-galactan II), O2a, and O2ac isolates (with 2c polysaccharide) (Whitfield et al., 1991, 1992). Recently, D-gal III has been identified as a component of the LPS of O1 and O2 strains; D-gal III is a product of conversion of the D-gal I disaccharide by the gmlABC operon (Stojkovic et al., 2017). More recently, multilocus sequence typing (MLST) has replaced O and K typing as the most common method used to type K. pneumoniae isolates (Diancourt et al., 2005).

The goal of this study was to determine the global diversity and distribution of O and K antigens on a large (>500) global collection of K. pneumoniae strains isolated from blood. This data will help to determine whether a glycoconjugate vaccine that targets a limited number of O antigens would provide coverage against the majority of K. pneumoniae bacteremic strains that are currently circulating worldwide. We are particularly interested in evaluating prevalence amongst blood culture isolates as our intended primary endpoint for a Phase 3 clinical trial would be prevention of systemic infections.

MATERIALS AND METHODS

Bacterial Strains
All K. pneumoniae isolates were collected from the blood of patients in 13 countries during 2005–2017 (Table 1). All isolates were identified using standard microbiological procedures. A random sample of K. pneumoniae strains collected from the Americas (United States and Ecuador), Europe (France, Denmark, and Sweden), Asia (Japan, Taiwan, Singapore, and Pakistan) and Africa (South Africa, DR Congo, and Mali), irrespective of antimicrobial resistance, was selected for analysis. However, isolates from Greece were inadvertently selected from a multi drug resistance collection. The number of isolates collected each year is shown for each country in Supplementary Figure S1. Altogether, 645 K. pneumoniae isolates were shipped to the Center for Vaccine Development and Global Health at the University of Maryland School of Medicine for subsequent testing and analysis. K. pneumoniae B5055 (O1), 7380 (O2), 390 (O3), Mich.61 (O4), 4425/51 (O5), 889 (O8), 1205 (O9), and 708 (O12) were used as reference strains for the Ørskov and Ørskov O typing scheme (Ørskov and Ørskov, 1984; Trautmann et al., 2004). K. pneumoniae strains were grown in animal product-free HY-Soy (HS) media (0.5% sodium chloride, 1% Hy-Soy (TEKnova, Hollister, CA, United States), 0.5% Hy-Yest 444 (Kerry Biosciences, Beloit, WI, United States) at 37°C. Genomic DNA was extracted using the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich, St. Louis, MO, United States) following the manufacturer’s instructions. DNA was extracted from 1.5 ml of overnight bacterial culture and eluted into 200 μl of elution solution (10 mM Tris–HCl, 0.5 mM EDTA, pH 9.0) and 2 μl of DNA was used for polymerase chain reaction (PCR).

Antimicrobial Susceptibility Test
Antimicrobial susceptibility was determined by the Kirby-Bauer disk diffusion method. The following antibiotics were tested in this study: amikacin, ampicillin/sulbactam, aztreonam, cefazolin, cefepime, ceftriaxone, ertapenem, gentamicin, levofloxacin, meropenem, piperacillin-tazobactam, and tetracycline. Antimicrobial impregnated disks (BD BBL™ Sensi-Disk™) were purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, United States). Antimicrobial susceptibility was determined by measuring the diameter of the zone of inhibition and the results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (M100-S29). MDR K. pneumoniae was defined as non-susceptible to at least one agent in three or more antibiotic classes. Non-MDR was defined as non-susceptible to less than three antimicrobial categories or fully susceptible (Magiorakos et al., 2012). Isolates from Greece were part of an MDR collection and were thus excluded from all analyses involving antimicrobial resistance to avoid bias from non-randomly selected strains.

Typing O Antigens and Genetic Analysis of D-Galactan Types
O typing was performed by PCR, using a method described by Fang et al. (2016) except that we did not differentiate O2ac from O2a. O1, O2, O3, O4, O5, O8, O9, and O12 were determined (identification of O7 which is rare was not done). Briefly, genomic DNA (>10 ng) was used as the template, Dreamtaq Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, United States) was used for PCR, with 0.4 μM primers and 2 mM Mg2+ in a final reaction volume of 50 μl. The following cycling conditions were used: 95°C for 10 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min, then a final extension at 72°C for 10 min. DNA fragment size was determined on a 1% agarose gel. D-galactan III-possessing strains were identified by screening O1 and O2 strains randomly selected from different regions for the gmlABC locus by PCR (Szijártó et al., 2016). The geographic distribution of O types was visualized using an online tool1.

K Typing
Two methods were used to K type K. pneumoniae: multiplex PCR and wzi gene sequencing. K1, K2, K5, K20, K54, and K57 were identified by multiplex PCR described by Turton et al. (2010). Briefly, multiplex PCR was performed using the Qiagen Multiplex PCR kit (Qiagen, Hilden, Germany) with 0.2 μM primers and 3 mM Mg2+ in a final reaction volume of 25 μl. Cycling parameters were 95°C for 15 min, followed by 35 cycles of 94°C for 30 s, 58°C for 90 s and 72°C for 90 s, and a final extension at 72°C for 10 min. Amplicons were separated on a 1.5% agarose gel.

The wzi gene was sequenced and K types determined as described by Brisse et al. (2013). PCR amplification of wzi was achieved by using PCR buffer containing 2 mM dNTPs, 0.4 μM primers, and 1 U of Green Taq DNA polymerase (Genscript, Piscataway, NJ, United States). Cycling conditions were as follows: 95°C for 3 min, followed by 40 cycles of 95°C for 30 s, 56°C for 45 s and 72°C for 40 s, and a final extension at 72°C for 5 min. After DNA purification using a QIAquick PCR Purification kit (Qiagen), amplicons were sequenced (Genewiz, South Plainfield, NJ, United States) and chromatograms were

1https://mapchart.net/
**TABLE 1 | Clinical Klebsiella pneumoniae isolates used in this study.**

| Region | Country         | Institute                                                      | Years       | Number of isolates |
|--------|-----------------|----------------------------------------------------------------|-------------|--------------------|
| Americas | United States | University of Maryland Medical Center                           | 2010–2015   | 107                |
|         | United States  | Duke University Medical Center                                   | 2008–2015   | 73                 |
|         | Ecuador         | Instituto Nacional de Investigación en Salud Publica. Universidad San Francisco de Quito | 2016       | 13                 |
| Europe  | France          | Saint-Antoine Hospital                                            | 2008–2011   | 11                 |
|         | Denmark         | Statens Serum Institute                                            | 2017        | 34                 |
|         | Sweden          | Karolinska University Hospital, Stockholm                         | 2015        | 25                 |
|         | Greece          | University General Hospital of Thessaloniki (AHEPA)              | 2014–2016   | 54                 |
| Asia    | Japan           | Nagoya University Graduate School of Medicine                    | 2010–2015   | 19                 |
|         | Taiwan          | Taipei Veterans General Hospital                                  | 2012–2013   | 6                  |
|         | Singapore       | Singapore General Hospital                                         | 2012–2016   | 25                 |
|         | Pakistan        | Aga Khan University                                               | 2015–2017   | 71                 |
| Africa  | South Africa    | National Institute for Communicable Diseases                     | 2011–2012   | 96                 |
|         | DR Congo        | University Hospital of Kinshasa                                   | 2015        | 83                 |
|         | Mali            | Center for Vaccine Development-Mali                               | 2005–2012   | 28                 |

reviewed and sequences trimmed using Geneious software (Biomatters Ltd., Auckland, New Zealand). The wzi alleles and K types were identified from the *K. pneumoniae* sequence typing database at http://bigdbs.web.pasteur.fr. Phylogenetic trees of wzi alleles were constructed using MEGA version 7.0 based on the neighbor-joining method (100 bootstrap replicates) and Jukes-Cantor distance (Brisse et al., 2013; Martin et al., 2016).

**Multilocus Sequence Typing (MLST) Analysis**

Multilocus sequence typing with seven genes (*gapA, infB, mdh, pgi, phoE, rpoB*, and *tonB*) was performed on strains according to the protocol described on the *K. pneumoniae* MLST website2 (Diancourt et al., 2005). The MLST database was used to assign alleles and sequence types (STs). Clonal complexes (CCs) were defined as groups for which MLST profiles showed only one allelic mismatch with at least one other member of the group (Bialek-Davenet et al., 2014).

**Statistical Analysis**

The proportion of susceptible versus resistant strains for each O type was analyzed by Chi-square analysis with Yate’s correction using GraphPad Prism version 6.05 software (GraphPad Software, San Diego, United States). The significance level was \( p < 0.05 \).

**RESULTS**

**Antimicrobial Resistance**

All 54 MDR isolates from Greece were resistant to ampicillin-sulbactam, cefazolin, cefepime, ceftriaxone, ertapenem, meropenem, and piperacillin-tazobactam (data not shown). These isolates were excluded from subsequent analyses (Supplementary Figure S2). High resistance rates to ampicillin-sulbactam (60.9%) and cefazolin (77.3%) were observed among 591 global isolates (see Supplementary Table S2). Resistance to carbapenems, which are used as a last resort for serious multidrug resistant infections (Meletis, 2016), was observed to be 19.3% (Table 2). The highest resistance rate against carbapenems was observed in Asia (51.2% of Asian isolates were resistant).

Overall, we observed 62.1% MDR globally (Table 2). Multidrug resistance varied by region with higher proportions observed in Africa (80.7%) and Asia (72.7%) than in the Americas (48.7%) or Europe (25.7%). In the United States, 53.3 and 38.4% of isolates collected from University of Maryland Medical Center and Duke University Medical Center were multidrug-resistant, respectively. MDR *K. pneumoniae* isolates were observed across Europe with the percentage ranging from 16 to 32.4%. The percentage of MDR isolates collected from Japan (31.6%) and Taiwan (16.7%) were lower than that of Singapore (56%) and Pakistan (94.4%).

**O Types and D-Galactan Types**

Among 645 *K. pneumoniae* isolates, 44.8% were O1, 19.5% were O2, 14.9% were O3, and 10.9% were O5. Collectively, these four O types accounted for 90.1% of *K. pneumoniae* strains in 13 countries (Figure 1). One O9 isolate in United States and 3 O12 isolates in two countries (United States and Sweden) were found while 14 O4 isolates were found globally. No O8 isolates were identified. Although we observed diverse profiles of O types between countries, the total percent of O1, O2, O3, and O5 isolates collectively ranged from 72% in Sweden to as high as 100% in Greece. We also evaluated the serotype distribution in the United States over time and found that the prevalence of O1, O2, O3, and O5 remained fairly stable between 2011 and 2015 (Supplementary Figure S3). We observed an increase in the percentage of O1 in the United States from 44% in 2011 to 50% in 2015 and in O5 from 2.2% in 2011 to 10.7% in 2015. In contrast, serotype O2 decreased from 20 to 3.6% though statistically significant differences were not reached due

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2www.pasteur.fr/mlst
## Table 2 | Antibiotic resistance of 591 Klebsiella pneumoniae isolated from blood.

| Region | Country | Number of isolates | Aminoglycosides | Penicillins + ß-lactamase inhibitors | Non-extended spectrum cephalosporins | Extended spectrum cephalosporins | Carbapenems | Monobactams | Fluoroquinolones | Tetracyclines |
|--------|---------|--------------------|-----------------|--------------------------------------|------------------------------------|-----------------------------------|----------------|-------------|----------------|---------------|
|        |         |                    |                 |                                      |                                    |                                   |                |             |                |               |
| Global |         | 591                | 266 (45.0)      | 382 (64.6)                           | 457 (77.3)                         | 338 (57.2)                       | 114 (19.3)    | 273 (46.2)  | 243 (41.1)    | 248 (42.0)    |
| Americas | United States | 107                | 29 (27.1)       | 59 (55.1)                            | 80 (74.8)                          | 52 (48.6)                        | 20 (18.7)     | 22 (20.6)   | 35 (32.7)     | 27 (25.2)     |
|         | United States | 73                 | 10 (13.7)       | 32 (43.8)                            | 51 (69.9)                          | 23 (31.5)                        | 9 (12.3)      | 4 (5.5)     | 17 (23.3)     | 19 (26.0)     |
|         | Ecuador   | 13                 | 9 (69.2)        | 10 (76.9)                            | 13 (100)                           | 9 (69.2)                         | 5 (38.5)      | 9 (69.2)    | 9 (69.2)      | 9 (69.2)      |
| Total   |         | 193                | 48 (24.9)       | 101 (52.3)                           | 144 (74.6)                         | 84 (45.5)                        | 34 (17.6)     | 35 (18.1)  | 61 (31.6)     | 94 (48.7)     |
| Europe  |         |                    |                 |                                      |                                    |                                   |                |             |                |               |
|         | France   | 11                 | 1 (9.1)         | 6 (54.5)                             | 3 (27.3)                           | 3 (27.3)                         | 0 (0)         | 3 (27.3)    | 1 (9.1)       | 5 (45.5)      |
|         | Denmark  | 34                 | 1 (2.9)         | 13 (38.2)                            | 23 (67.6)                          | 7 (20.6)                         | 3 (8.8)       | 3 (8.8)     | 6 (17.6)      | 7 (20.6)      |
|         | Sweden   | 25                 | 1 (4)           | 7 (28)                               | 8 (32)                             | 1 (4)                            | 1 (4)         | 1 (4)       | 3 (12)        | 4 (16)        |
| Total   |         | 70                 | 3 (4.3)         | 26 (37.1)                            | 34 (48.6)                          | 11 (15.7)                        | 4 (5.7)       | 7 (10)      | 10 (14.3)     | 15 (21.4)     |
| Asia    | Japan    | 19                 | 1 (5.3)         | 8 (42.1)                             | 9 (47.4)                           | 4 (21.1)                         | 0 (0)         | 3 (15.8)    | 4 (21.1)      | 6 (31.6)      |
|         | Taiwan   | 6                  | 1 (16.7)        | 2 (33.3)                             | 3 (50)                             | 0 (0)                            | 2 (33.3)      | 0 (0)       | 1 (16.7)      | 2 (33.3)      |
|         | Singapore| 25                 | 10 (40.0)       | 16 (64.0)                            | 21 (84.0)                          | 11 (44.0)                        | 7 (28.0)      | 8 (32.0)    | 12 (48.0)     | 10 (40.0)     |
|         | Pakistan | 71                 | 54 (76.1)       | 65 (81.5)                            | 69 (87.2)                          | 64 (80.1)                        | 53 (74.6)     | 61 (85.9)  | 65 (91.5)     | 41 (57.7)     |
| Total   |         | 121                | 66 (54.5)       | 91 (75.2)                            | 102 (84.3)                         | 79 (65.3)                        | 62 (61.2)     | 72 (59.5)  | 82 (67.8)     | 59 (48.8)     |
| Africa  | South Africa | 96                | 67 (69.8)       | 80 (83.3)                            | 86 (89.6)                          | 76 (79.2)                        | 8 (8.3)       | 73 (76)    | 50 (52.1)     | 41 (42.7)     |
|         | DR Congo | 83                 | 63 (75.8)       | 64 (77.1)                            | 70 (84.3)                          | 68 (81.9)                        | 5 (6.0)       | 68 (81.9)  | 31 (37.3)     | 66 (79.5)     |
|         | Mali     | 28                 | 19 (67.9)       | 20 (71.4)                            | 21 (75.0)                          | 20 (71.4)                        | 1 (3.6)       | 18 (64.3)  | 9 (32.1)      | 12 (42.9)     |
| Total   |         | 207                | 149 (72.0)      | 164 (79.2)                           | 177 (85.5)                         | 164 (79.2)                       | 14 (6.8)      | 159 (76.8) | 90 (43.5)     | 119 (57.5)    |

^n| Aminoglycosides (aminoglycosides); piperacillin/tazobactam and ampicillin/subactam (penicillins + ß-lactamase inhibitors); ceftriaxone and cefepime (extended spectrum cephalosporins, the 3rd and 4th generations); meropenem and ertapenem (carbapenems); aztreonam (monobactams); levofloxacin (fluoroquinolones); tetracycline (tetracycline) were used in this study.  
| Isolates collected from Greece were not included.  
| MDR defined as resistant to at least three antibiotic classes.  
| Isolates were obtained from University of Maryland Medical Center.  
| Isolates were obtained from Duke University Medical Center.  
| Isolates were obtained from University of Maryland Medical Center.  
| Isolates were obtained from Duke University Medical Center.  


Some K. pneumoniae O1 and O2 strains have been shown to possess a modified galactan O-antigen type (Stojkovic et al., 2017). We screened 199 O1 and O2 isolates for the presence of gmlABC which encodes this D-galactan III antigen. We detected gmlABC in 49.7% of isolates tested. Our data suggests that D-galactan I is more common in Europe than in other regions (Table 3).

To determine if particular serotypes were associated with antibiotic resistance, we evaluated the serotype distribution amongst susceptible and non-susceptible strains for each antibiotic class and for MDR versus non-MDR strains. The serotype distribution observed among 367 MDR isolates was 49% for O1, 17% for O2, 11% for O3, and 14% for O5, respectively (Figure 2A). There were significantly more O1 and fewer O3 strains in the MDR group compared to the non-MDR group ($p < 0.05$). The percentage of O serotypes in the susceptible and non-susceptible groups to each antibiotic is shown in Figure 2B. There were significantly more O1 strains that were non-susceptible to aminoglycosides and tetracycline compared to susceptible ($p < 0.05$). Likewise, there were more O2 strains that were not susceptible to extended spectrum cephalosporins, carbapenems, monobactams, and fluoroquinolones than susceptible. There were more O5 strains that were non-susceptible to aminoglycosides and...
TABLE 3 | Distribution of D-galactan I (gmlABC-negative) and III (gmlABC-positive) in O1 and O2 Klebsiella pneumoniae in 13 countries.

| Region | No. of gmlABC-negative strains (%) | No. of gmlABC-positive strains (%) | ND\(^c\) | Total |
|--------|-----------------------------------|-----------------------------------|---------|-------|
| Americas | 24 (48%) | 26 (52%) | 0 (0%) | 50 (100%) |
| Europe | 31 (62%) | 19 (38%) | 0 (0%) | 50 (100%) |
| Asia | 20 (40.8%) | 27 (55.1%) | 2 (4.1%) | 49 (100%) |
| Africa | 20 (40%) | 27 (54%) | 3 (6%) | 50 (100%) |
| Total | 95 (47.8%) | 99 (49.7%) | 5 (2.5%) | 199 (100%) |

\(^a\)As determined by amplification of a 2.1-kb PCR product. \(^b\)As determined by amplification of a 5-kb PCR product. \(^c\)ND, not determined (no amplification).

DISCUSSION

The objective of this study was to determine whether a vaccine that targets *K. pneumoniae* O1, O2, O3, and O5 antigens would provide coverage against the majority of bacteremic strains circulating worldwide. In this study, we examined rates of antibiotic resistance, the distribution and diversity of O antigens, K antigens, and STs and among 645 invasive clinical isolates collected from 13 countries. This is the largest study of invasive *K. pneumoniae* that has been performed to date with isolates coming from diverse geographic locations.

Carbapenems have been considered as a treatment of last choice for multidrug-resistant *K. pneumoniae* (Meletis, 2016; Pennini et al., 2017). Recently, the increased emergence of *K. pneumoniae* resistant to those antibiotics has become a serious problem worldwide. We observed the high rate of non-susceptibility to carbapenems (Table 2), suggesting that antibiotic treatment options for multidrug-resistant *K. pneumoniae* are becoming limited. We observed higher multidrug resistance (62.1%) than Hackel et al. (2016) who found 30.1% MDR out of 9,098 *K. pneumoniae* isolates collected in 2012–2014 from 176 geographic sites. These strains were isolated predominantly from intra-abdominal infections, urinary tract infections, skin and soft tissue infections and lower respiratory tract infections. Unlike our collection, only 3.8% of the isolates were from blood. Further, the higher proportion of multidrug resistant organisms that we observed in our study may be due to inclusion of isolates from sub-Saharan Africa and South Asia which show high rates of MDR (Investigators of the Delhi Neonatal Infection Study (DeNIS) collaboration, 2016; Henson et al., 2017; Musicha et al., 2017). Hackel et al. described isolates from South Africa but no other African countries and no isolates from India, Bangladesh or Pakistan (Hackel et al., 2016).

We found that 90.1% of the *K. pneumoniae* isolates were O1, O2, O3, or O5, consistent with previous reports (Figure 1; Trautmann et al., 1997; Follador et al., 2016). O1 and O2 *K. pneumoniae* isolates have been considered important pathogens due to their high prevalence, virulence and rate of antibiotic resistance (Fang et al., 2016; Szijártó et al., 2016; Pennini et al., 2017). While D-galactan I antigen was first identified as a repeat unit of O polysaccharide in O1 and O2 isolates, some O1 and O2 strains were recently reported to possess a modified D-galactan I, called D-galactan III (Whitfield et al., 1992; Szijártó et al., 2016; Stojkovic et al., 2017). In this study, 49.7% isolates carried the gmlABC operon which is associated with D-gal III synthesis. Similarly, Szijártó et al. (2016) reported that 40% of O1 isolates and 68% of O2 isolates possess the gmlABC operon (Szijártó et al., 2016). These results imply that an O-polysaccharide-based vaccine would need to ensure that antibodies recognize both the D-gal III epitope as well as the D-gal I epitope to achieve maximal coverage (Stojkovic et al., 2017).

Although O1 and O2 strains are the most common serotypes globally (64.3%), 25.7% of strains tested were determined to be O3 or O5. These findings suggest that vaccines for preventing infection caused by O3 and O5 *K. pneumoniae* are also necessary. No O8 isolate was found among 645
**FIGURE 2** | The prevalence of O types in invasive *Klebsiella pneumoniae* that are susceptible or resistant to antibiotics. (A) The percentage of O types amongst non-MDR (inner pie chart) and MDR isolates (outer pie chart). (B) The percentage of O types amongst isolates susceptible (inner pie chart) and non-susceptible (outer pie chart) to each antibiotic. Statistically significant differences (*P* ≤ 0.05) between susceptible and non-susceptible isolates are indicated by asterisks (*). Isolates collected from Greece were not included.

*K. pneumoniae* in line with that seen in studies by other investigators (Follador et al., 2016). It should be noted that *K. pneumoniae* O1 and O8 O polysaccharide are serologically similar and O1 OPS would likely protect against O8 strains (Trautmann et al., 1997). O12 isolates have been isolated from several locations in Europe including Spain, Denmark, and United Kingdom (Hansen et al., 1999; Follador et al., 2016). We found only 3 O12 isolates from United States and Sweden. Thus, targeting O1, O2, O3, and O5, but not O12, would be important for development of a broad spectrum vaccine against *K. pneumoniae*. In the current study, the most common O type was O1, regardless of antibiotic resistance, which is associated with high bacterial dissemination and colonization into organs (Hsieh et al., 2014). Pennini et al. (2017) reported that O2 was the dominant serotype of MDR *K. pneumoniae* and associated with β-lactam and carbapenem-resistance (Pennini
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**FIGURE 3** | Distribution of K types in invasive *Klebsiella pneumoniae* isolates in the multi-country collection. (A) K types by multiplex PCR among 474 isolates. The numbers on top of the bars represent the number of isolates belonging to each K type. (B) Phylogenetic tree for *wzi* sequence of 265 invasive randomly selected strains. The scale bar represents the amount of genetic change; 0.01 equals 1 change per 100 nucleotide sites. The categories of O serotypes and the geographical origins are represented by colors as indicated.

et al., 2017). In our study, although O2 was associated with resistance to extended spectrum cephalosporins, carbapenems, monobactams, and fluoroquinolones (Figure 2B), O1 was the dominant serotype of MDR *K. pneumoniae* and associated with resistance to aminoglycosides and tetracycline. O5 strains were associated with non-susceptibility to aminoglycosides, and monobactams. In the Pennini et al. (2017) study, 57.7% of isolates were from respiratory sites and only 9.2% were from blood/peritoneum. All of the isolates examined in the current study were from blood culture. In addition, the distribution of isolates from geographic sites was different in the Pennini et al. (2017) study compared to our study. A higher proportion of isolates were from Europe (38% for Pennini et al. versus 19% for our study) and a lower proportion of strains were from Africa (5% for Pennini et al. versus 32% for our study). This may explain the difference in serotype distribution that we observed amongst MDR strains compared to Pennini et al. (2017). Collectively, the data suggests that it is important to prevent *K. pneumoniae* O1 infections due to their high prevalence and virulence and serotypes O2 and O5 due to their association with antibiotic resistance.

### TABLE 4 | Distribution of sequence types in O1:K2 *Klebsiella pneumoniae* isolates.

| Region | Isolate | Year | ST |
|--------|---------|------|----|
| Americas | 12-00541 | 2012 | 14 |
| | 15-02188 | 2015 | 14 |
| | 15-00479 | 2015 | 14 |
| Asia | NUHS204 | 2015 | 25 |
| | NUHS279 | 2015 | 25 |
| | EC1911 | 2016 | 14 |
| Africa | 7032 | 2012 | 25 |
| | 7029 | 2012 | 14 |
| | 4630027 | 2012 | 66 |
| | 4897/4 | 2015 | 25 |

ST, sequence type.
A high prevalence of K2 isolates among 519 invasive strains was similarly observed by Paczosa and Mecsas (2016). K24 was the most common capsule type in our study. The hypervirulent K1 capsule type (Siu et al., 2011) was found amongst isolates collected from United States, Taiwan, South Africa, DR Congo, and Pakistan by wzi capsular typing. Diverse K types were identified as has been previously reported (Cryz et al., 1986b), indicating that a vaccine that targets only 4 O antigens would be less difficult and less expensive to construct and provide broad coverage compared to a vaccine that targets the capsular polysaccharide (that would need 24 K antigens to provide the same coverage).

Our data showed that clonally diverse K. pneumoniae caused bacteremia in patients in 13 countries. Additionally, there was no association between particular O serotypes and sequence types. Therefore, MLST data cannot be used as a proxy for O typing. Furthermore, a strategy to target antigens specific for certain STs might be insufficient to prevent infections caused by diverse lineages of K. pneumoniae.

In summary, we determined that there are 4 major O antigen types among 645 strains and 49 K antigens among 519 strains collected from 13 countries. In addition, diverse STs and a high rate of MDR K. pneumoniae was determined. Therefore, we believe that a K. pneumoniae O antigen-based vaccine would offer an excellent alternative strategy to a vaccine based on capsular polysaccharide which has previously been proposed, developed and evaluated in a Phase 1 study (Edelman et al., 1994). Furthermore, by preventing K. pneumoniae infections from occurring in the first place, this will eliminate the need for antibiotics and will therefore remove selective pressure for resistance (Klugman and Black, 2018). Additionally, prevention is far superior to treatment as those patients who survive sepsis suffer multiple long-term complications. Our data suggests that our proposed quadrivalent vaccine (Hegerle et al., 2018) could potentially prevent 90.1% of K. pneumoniae bacteremic infections worldwide.

**DATA AVAILABILITY STATEMENT**

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

**AUTHOR CONTRIBUTIONS**

ST, RS, and AC designed the study. MC, ST, RS, and AC performed the analysis and wrote the manuscript. MT, JKK, SM, JT, VF, AA, ET, DD, FM, KK, EC, YS, Y-TL, SaS, OP, AS-M, JJ, and OL collected the strains. NH, JN, SuS, SJ, SN, ShS, JP-B, and JS performed the assays. All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.01249/full#supplementary-material

**FIGURE S1** | The number of K. pneumoniae isolates analyzed by year and country.

**FIGURE S2** | The number of K. pneumoniae isolates tested by each method.

**FIGURE S3** | Trends in O antigens of invasive K. pneumoniae collected from University of Maryland Medical Center in 2011–2015.

**FIGURE S4** | Distribution of K types by wzi gene sequencing among 519 isolates. Allele# represents wzi alleles which have been assigned an allele number by the wzi database at http://bigd.pasteur.fr but which do not correspond to a K type.

**TABLE S1** | Sequence types of 39 isolates with O1, O2, O3, O4, and O5 antigens.

**TABLE S2** | Resistance of 591 K. pneumoniae isolated from blood to 12 different antibiotics.
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**Conflict of Interest:** ST, RS, and AC are holders of US patent 9,988,426 titled “Broad spectrum conjugate vaccine to prevent *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* infections.”

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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