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Characterization of Pre-Antibiotic Era *Klebsiella pneumoniae* Isolates with Respect to Antibiotic/Disinfectant Susceptibility and Virulence in *Galleria mellonella*

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The EGD Murray collection consists of approximately 500 clinical bacterial isolates, mainly *Enterobacteriaceae*, isolated from around the world between 1917 and 1949. A number of these "Murray" isolates have subsequently been identified as *Klebsiella pneumoniae*. Antimicrobial susceptibility testing of these isolates showed that over 30% were resistant to penicillins due to the presence of diverse bla_{SHV} β-lactamase genes. Analysis of susceptibility to skin antiseptics and triclosan showed that while the Murray isolates displayed a range of MIC/minimal bactericidal concentration (MBC) values, the mean MIC value was lower than that for more modern *K. pneumoniae* isolates tested. All Murray isolates contained the cation efflux gene *cepA*, which is involved in disinfectant resistance, but those that were more susceptible to chlorhexidine were found to have a 9- or 18-bp insertion in this gene. Susceptibility to other disinfectants, e.g., H_2O_2, in the Murray isolates was comparable to that in modern *K. pneumoniae* isolates. The Murray isolates were also less virulent in *Galleria* and had a different complement of putative virulence factors than the modern isolates, with the exception of an isolate related to the modern lineage CC23. More of the modern isolates (41% compared to 8%) are classified as good/very good biofilm formers, but there was overlap in the two populations. This study demonstrated that a significant proportion of the Murray *Klebsiella* isolates were resistant to penicillins before their routine use. This collection of pre-antibiotic era isolates may provide significant insights into adaptation in *K. pneumoniae* in relation to biocide susceptibility.

Since the end of the last century, there has been a growing concern about the rise of infections caused by multidrug-resistant (MDR) Gram-negative *Enterobacteriaceae*. *Klebsiella* spp., and in particular *Klebsiella pneumoniae*, are opportunistic pathogens of increasing importance. The recent outbreaks of carbapenemase-producing *K. pneumoniae* are of particular concern (1–3). Modern *K. pneumoniae* strains are intrinsically resistant to penicillins and can acquire resistance to third- and fourth-generation cephalosporins or carbapenems owing to production of plasmid-borne extended-spectrum β-lactamases (ESBLs) or carbapenemases, e.g., KPC, respectively (4, 5). Often these resistance genes are found on the same plasmid as other antibiotic resistance genes, e.g., those for aminoglycoside resistance, giving rise to MDR strains that can spread rapidly. Indeed the U.S. Centers for Disease Control and Prevention (CDC) has now identified carbapenemase-producing *Enterobacteriaceae* (CPE) as one of its three most urgent antibiotic resistance threats (6).

The EGD Murray collection consists of over 500 (mainly *Enterobacteriaceae*) strains isolated from many parts of the world, including Europe, the Middle East, northern Russia, North America, and India (7). These strains were isolated predominately from a variety of human infections pre-1949, which was before many antibiotics were available. Penicillin was discovered in 1928 (8) but came into more widespread use after mass production techniques were utilized in 1943. The first description of a penicillinase was made in 1940 (9). Several isolates in the Murray collection have been identified as *K. pneumoniae*, and studies on such pre-antibiotic era isolates are of significant value in understanding the development of antimicrobial resistance and other phenotypic changes in this important pathogen.

This study aimed to characterize the "Murray collection" *Klebsiella* isolates in terms of their susceptibility to antibiotics, disinfectants, and antiseptics and their virulence in a *Galleria mellonella* infection model. We showed that several Murray isolates were resistant to penicillins due to a bla_{SHV} gene and that high levels of susceptibility to antiseptics, particularly chlorhexidine, were associated with a short (9- or 18-bp) insertion in the *cepA* (*fieF*) gene. Murray isolates, with one exception, showed poor virulence in the *G. mellonella* infection model.

MATERIALS AND METHODS

Bacterial isolates and culture conditions. The Murray isolates consisted of 37 isolates noted as being *Klebsiella* in original records and strain NCTC 5054, isolated in 1937. The modern strains were comprised of 34 previously described isolates (10) and a further 5 isolates from 2007 to 2012.

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TABLE 1 Presence of potential virulence factors in *Klebsiella* isolates

Era and capsular type	No. of isolates	% positive for:									
		rmpA	*wcaG*	*fimH*	*uge*	*iutA*	*kfu*	*ureA*	*mrkD*	*wabG*	bla_{SHV}
Murray	38	76.3	63.2	94.7	86.8	5.3	73.7	100	13.2	100	34.2
K1	24	100	100	100	83.3	8.3	100	100	4.2	100	4.2
Non-K1	14	35.7	0	85.7	92.9	0	28.5	100	28.6	100	85.7
Modern, various[a]	39	23.1	20.5	89.7	94.9	20.5	35.9	100	87.2	100	97.4

[a] No predominant capsular type was identified in this population.

(Full isolation details are shown in Table S1 in the supplemental material.) For isolate identification, Vitek2 GN (bioMérieux) and matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) (11) analysis was used. All isolates were grown in TSB broth with aeration or on TSB agar plates at 37°C, unless otherwise stated.

Phenotype assessment. (i) Antibiotic/disinfectant susceptibility testing. MICs were performed using a broth microdilution method. All cultures were incubated at 37°C for antibiotics and antiseptics, but at room temperature (18 to 22°C) for disinfectants, for approximately 20 h. For minimal bactericidal concentration (MBC) testing, 25 μl of suspension was removed from each well of the MIC microtiter plate where no bacterial growth was observed, along with appropriate controls (i.e., the two wells immediately below the MIC where growth was observed). This was spotted on to tryptic soy agar (TSA) plates and incubated at 37°C for 24 h.

(ii) Analysis of biofilm formation. The ability of *Klebsiella* isolates to form biofilms was tested using a modification of the Calgary biofilm method (12), as previously published (13). A_{570} was measured using a FLUOstar Omega plate reader (BMG Labtech). Biofilm formation was scored relative to the absorbance value (+++, ≥0.4; ++, 0.2 to 0.4; +, 0.1 to 0.2; and +/−, < 0.1).

(iii) *G. mellonella* killing assays. Wax moth larvae (*G. mellonella*) were purchased from Livefood UK Ltd. (Rooks Bridge, Somerset, United Kingdom) and were maintained on wood chips in the dark at 15°C until used. Bacterial infection of *G. mellonella* was as previously described (14). All experiments were carried out in triplicate.

(iv) bla_{SHV} expression analysis. To ascertain whether the bla_{SHV} gene from M43 was functional, the full-length gene and its promoter was amplified using the SHVN001 (TAATGGATCCTGACCAACAGCCCGTCC GCCTTACC) and SHVN003 (TAATTCTAGAGAGGTGCTACGGGCC GGATAACG) primers. The bla_{SHV} gene and its promoter from M109 were also amplified using SHVN001 and SHVN002 (TAATTCTAGAAA CGCGCGCGGCCACCGCCGGG) as a control. Both fragments were digested with BamHI and XbaI and ligated into pACYC-184. *Escherichia coli* TOP-10 cells were transformed with the plasmid and colonies selected initially on LB-chloramphenicol (30 μg/ml); resultant colonies were restreaked on LB-ampicillin (50 μg/ml) plates to check for expression and activity of the bla_{SHV} genes.

Genotype analysis. (i) Detection of virulence genes in *Klebsiella*. PCR was performed on all isolates to amplify the virulence genes listed in Table 1. Previously described specific primers for K1 and K2 capsular type, *rmpA*, and *wcaG* (15), capsular type K3 (16), *fimH*, *uge*, *wabG*, *kfu*, *ureA*, *allS*, and *mrkD* (17), and aerobactin (*iutA*) (10) were used in this study.

(ii) Phylogenetic analysis of Murray isolates and bla_{SHV} gene sequences. Whole-genome sequencing of Murray isolates was performed on an Illumina (HiSeq) instrument. Sequencing data are available at the European Nucleotide Archive (accession numbers are in Table S1 in the supplemental material). The sequencing data were assembled using Velvet (18) and the core genome determined as genes where an orthologue (as defined by 97% sequence identity using BLAST) (19) was present once in every isolate in the analysis. A maximum-likelihood phylogenetic tree was constructed using RAxML (GTR + Γ_4 model) (20) based on a concatenated multiple-sequence alignment of the 55,961 variant sites from 2,945 core genes comprising 2,777,973 bp.

(iii) bla_{SHV} and *cepA* gene sequences. The nucleotide coding bla_{SHV} and *cepA* sequences (identified by both BLAST comparisons of sequence data and PCR) were aligned using MUSCLE (21).

Statistical analysis. Confirmed colony variants were excluded from proportion calculations. Mean MIC/MBC values for antiseptics and triclosan for the Murray and modern groups of isolates were calculated and *P* values determined using the Student unpaired *t* test to show whether the difference in values between the two groups was significant ($P < 0.01$).

RESULTS

General properties of the Murray *Klebsiella* isolates. The Murray isolates are human isolates from a variety of clinical conditions, e.g., sputum from pneumonia patients or blood clots (see Table S1 in the supplemental material). All *Klebsiella* isolates were verified as *K. pneumoniae* by MALDI-TOF analysis, except M344, which was identified as *Raoultella ornithinolytica* and excluded from further analysis. All *Klebsiella* isolates were further verified using primers specific to the *Klebsiella gapA* and *rpoB* genes.

The *Klebsiella* Murray isolates were analyzed with the Vitek2 and were again identified as either *Klebsiella pneumoniae* subsp. *pneumoniae* or *Klebsiella pneumoniae* subsp. *ozaenae*, and this revealed biochemical differences between the Murray and modern isolates, e.g., β-galactosidase and β-xylosidase production (see Table S1 in the supplemental material).

Phylogenetic analysis based on whole-genome sequencing of the Murray isolates revealed that there was considerable diversity, although a large clade of isolates of the same sequence type (ST82) existed (Fig. 1). Several isolates had been recorded as being colony variants from the same patient (see Table S1 in the supplemental material), which was confirmed by the phylogenetic analysis, with the exception of isolates M295 and M296 (from a surgical wound site and stool sample of a single patient, respectively), which were found to be distinct (Fig. 1).

Correlation of population structure with virulence determinants. Capsular types K1 to -3 were looked for using PCR. Isolates that were ST82 were positive for capsular type K1, a further two isolates (M585 and M586) were capsular type K3, and no isolates were capsular type K2. The Murray isolates were also examined for certain potential virulence genes, which had been previously examined in the majority of modern isolates (10) (Table 1). Splitting the Murray isolates into capsular K1 and non-K1 groups, those that were K1 type were also mucoid (*rmpA* positive) and contained *wcaG*, *fimH*, *kfuB*, *ureA*, and *wabG*. Many (>80%) of the Murray K1-type isolates were also positive for the presence of *uge*, but only one isolate (M109) contained *mrkD* and *iutA*. M109 was also positive for *allS*, an indicator for CC23, and was subsequently identified as ST23. For the non-K1 capsular Murray isolates, all were positive for the presence of *fimH* and *ureA* and many contained *uge* and *wabG*, but the presence of *rmpA*, *kfuB* and *mrkD*

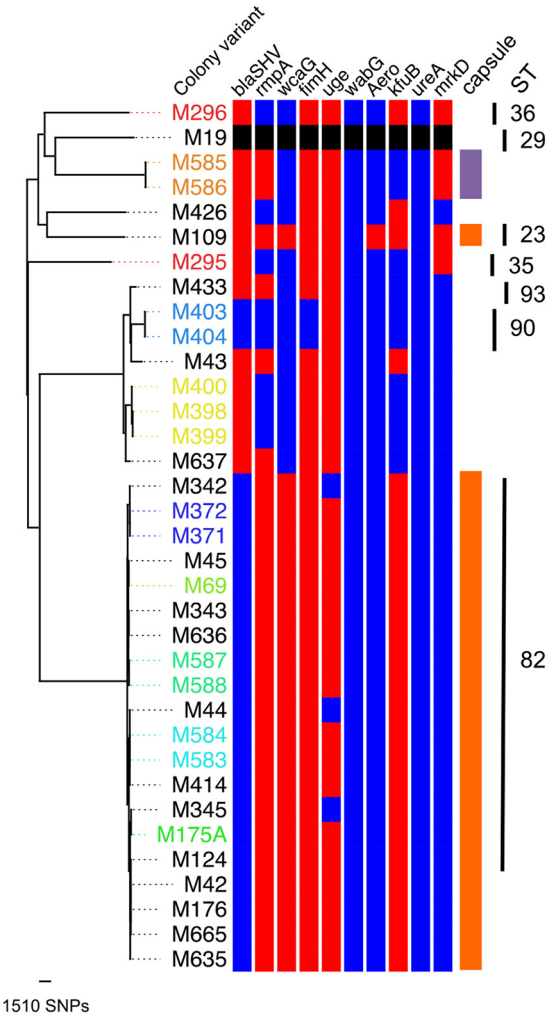

FIG 1 Genetic diversity in the Murray collection *Klebsiella* strains. The midpoint-rooted phylogenetic tree shows the phylogenetic relationships of taxa based on core genome. Taxon names of isolates noted as colony variants are colored similarly (noncolony variants are in black). The adjacent columns show the presence (red) and absence (blue) of bla_{SHV} genes and virulence-associated genes (black fields denote untested isolates). The presence of K1 (orange) and K3 (purple) capsular types is shown adjacent, and known sequence types (STs) are also shown.

was detected in few isolates. No isolates were found to contain *wcaG* and *iutA*.

Phenotype assessment. Biofilm formation ability was tested using the Calgary screening method, and the majority of Murray isolates were found to be poor biofilm formers (+/− or +) (see Fig. S1 in the supplemental material). For the modern isolates, biofilm formation was more mixed, with a range of biofilm formation shown (see Fig. S1 in the supplemental material). The virulence of these isolates in *Galleria* was also assessed and compared to that of modern isolates (Fig. 2). All Murray isolates except M109 were poorly virulent at an input of 1×10^5 CFU. Infection with M109 at this dose showed complete killing, and at a lower dose (1×10^4 CFU), approximately 50% *Galleria* mortality was observed. Virulence in *Galleria* was not linked to the growth rate of *Klebsiella* strains. Although several of the Murray isolates showed a reduced growth rate compared to that of more modern isolates (MGH 78578 and NCTC 13368, which are both virulent in *Galleria*), there were numerous isolates which were comparable (see Table S1 in the supplemental material). Again, when the growth rates of several modern isolates of differing virulence in *Galleria* were compared with each other, there was no correlation between growth rate and virulence in *Galleria*.

Several Murray isolates are resistant to penicillins. To determine antibiotic resistance, the MICs for 19 antibiotics was determined for all Murray isolates using a broth dilution method (Table 2). Antibiotics tested include representatives from all major classes, e.g., aminoglycosides and penicillins. All Murray isolates were susceptible to the majority of antibiotics tested, including cephalosporins, carbapenems, quinolones, and certain miscellaneous antibiotics, e.g., colistin. However, several isolates showed high levels of resistance (MIC of >64 μg/ml) to the penicillin class of antibiotic (e.g., ampicillin and piperacillin), though the addition of a β-lactam inhibitor (e.g., tazobactam or clavulanic acid) rendered them susceptible. This suggested the presence of a β-lactamase in several isolates.

Resistance to penicillins is due to the presence of diverse bla_{SHV} genes. All penicillin-resistant Murray isolates were investigated for the presence of bla_{TEM} and bla_{SHV} genes. No bla_{TEM} genes were detected, but all resistant *Klebsiella* isolates contained a bla_{SHV} gene. The *R. ornithinolytica* M344 isolate was also resistant to penicillin and ampicillin due to a bla_{ORN-1O} gene. *In silico* detection of bla_{SHV} genes also revealed that one strain (M43) contained a full-length bla_{SHV} gene but was phenotypically suscepti-

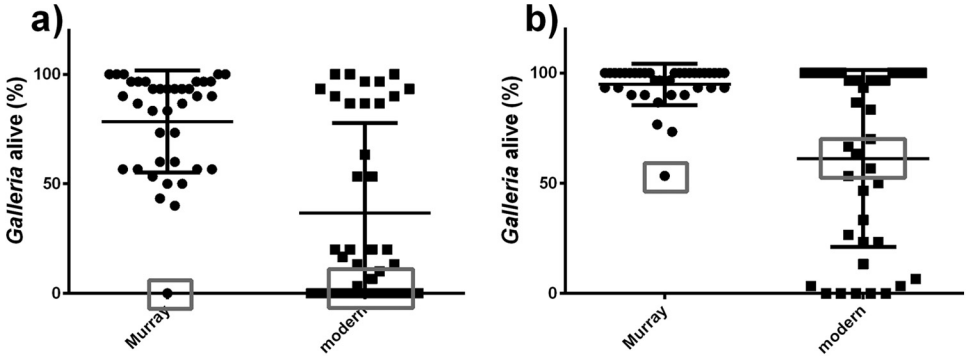

FIG 2 Virulence of *K. pneumoniae* in *G. mellonella* after 24 h. Groups of 10 larvae were challenged with 1×10^5 (a) and 1×10^4 (b) CFU of different strains of Murray and modern *Klebsiella* spp. The proportion of larvae alive at 24 h postinfection is shown, with individual points being the mean of independent triplicates and error bars showing the standard deviation of the mean of all points. Data for virulence of M109 and related modern CC23 isolates are highlighted by gray boxes.

TABLE 2 Resistance in *K. pneumoniae* Murray and modern isolates to clinical frontline antibiotics

Antibiotic class	Antibiotic[a]	MIC (μg/ml)					
		Murray isolates			Modern isolates		
		Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
Penicillins	AMP	0.5–>64	1	>64	64–>64	>64	>64
	PIP	0.25–>64	1	>64	8–>64	>64	>64
	TZP	0.25–8	1	4	1–>64	16	>64
	PEN	1–>64	4	>64	64–>64	>64	>64
	AMX	0.5–>64	1	>64	64–>64	>64	>64
	AMC	0.5–4	0.5	4	1–>64	32	>64
Cephalosporins	CTX	≤0.5	≤0.5	≤0.5	≤0.5–>512	>512	>512
	CAZ	≤0.5–1	≤0.5	≤0.5	≤0.5–>512	512	>512
Carbapenems	IMP	0.125–1	0.125	0.5	0.125–>64	0.5	64
	MEM	≤0.06–1	≤0.06	≤0.06	≤0.06–>64	≤0.06	64
Other β-lactam	ATM	≤0.06–0.25	≤0.06	0.125	≤0.06–>64	>64	>64
Quinolones	CIP	≤0.5	≤0.5	≤0.5	≤0.5–256	2	64
	LVX	≤0.5	≤0.5	≤0.5	≤0.5–64	1	64
Aminoglycosides	AMK	1–8	2	4	4–>512	32	128
	GEN	0.5–4	2	2	2–>512	16	512
	TOB	1–4	2	4	2–>512	128	>512
Miscellaneous	CHL	1–4	2	2	1–>512	4	512
	CST	0.5–2	1	2	0.25–64	2	2
	TMP	0.125–4	0.5	2	≤0.5–>512	>512	>512

[a] AMP, ampicillin; PIP, piperacillin; TZP, piperacillin-tazobactam; PEN, penicillin; AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime; IMP, imipenem; MEM, meropenem; ATM, aztreonam; CIP, ciprofloxacin; LVX, levofloxacin; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; CHL, chloramphenicol; CST, colistin; TMP, trimethoprim.

ble to penicillin class antibiotics. Interrogation of the surrounding genome sequence revealed no unusual base pair changes in the upstream promoter region or the downstream terminator region. Cloning the bla_{SHV} gene and its native promoter onto a plasmid for expression in *E. coli* revealed that the gene was unable to confer resistance to ampicillin, piperacillin, and penicillin beyond background levels. However, when this strain was passaged onto plates containing ampicillin (50 μg/ml), growth of colonies was observed. Subsequent sequencing of the bla_{SHV} gene in these clones revealed coding mutations or nucleotide changes in the promoter region around the ribosome binding site (RBS). These mutants showed increased levels of resistance to ampicillin and piperacillin (256 to >512 μg/ml for ampicillin and 8 to >512 μg/ml for piperacillin). The MICs with the "parental" bla_{SHV} gene are 4 to 8 μg/ml for ampicillin and 2 μg/ml for piperacillin. These strains were not resistant to ceftazidime, indicating that these mutations are not in ESBL-type β-lactamase genes.

The bla_{SHV} genes from the Murray isolates and several modern isolates ($n = 22$) were sequenced to determine what type of bla_{SHV} gene was present. Phylogenetic analysis of the nucleotide sequences of the bla_{SHV} genes revealed sequence diversity even within the same bla_{SHV} type (see Table S2 in the supplemental material). As expected, the true colony variants had identical bla_{SHV} sequences [i.e., not M295 (bla_{SHV-11}) and M296 (bla_{SHV-33})]. Overall, there were 10 different bla_{SHV} sequences present in *Klebsiella* Murray isolates. In comparison to the nucleotide sequences of the bla_{SHV} genes in the modern strains, bla_{SHV-11} from M109 was 100% identical to the bla_{SHV} genes in M6, 1084, NTUH-K2044, and TW3, which are all members of CC23 (data not shown). In the Murray isolates, bla_{SHV-11} was the most frequently detected, but other class 2b bla_{SHV}-types (e.g., bla_{SHV-1} and bla_{SHV-33}) were also present. Novel bla_{SHV} types were also detected in isolates M433, M43, and M426. The modern strains analyzed contained a mixture of non-ESBL SHV-types, of which again bla_{SHV-11} appeared to be the most common, and ESBL SHV types (e.g., bla_{SHV-12}, bla_{SHV-18}).

Several Murray isolates showed increased susceptibility to tested skin antiseptics but not disinfectants. The susceptibility of the Murray isolates to several different types of disinfectants, including hydrogen peroxide-, chlorine-, and quaternary ammonium-based disinfectants, was tested. Susceptibility to hydrogen peroxide and ethanol alone, which are components in different biocide formulations, was also verified. These results were compared with those for modern *Klebsiella* isolates to ascertain whether the modern strains showed decreased susceptibility. There was no significant decrease in susceptibility toward these disinfectants or chemicals, with MIC values for the Murray and modern isolates showing negligible difference and the measured MIC values being within narrow ranges (e.g., for H_2O_2, 0.03 to 0.06% [median value, 0.03%] for the Murray isolates and 0.03 to 0.06% [median value, 0.06%] for the modern isolates) (see Table S1 in the supplemental material). The exception was triclosan, where the Murray isolates had a lower range (0.007 to 0.5 μg/ml [mode, 0.06 μg/ml; median, 0.06 μg/ml]) than the modern isolates (range, 0.125 to >4 μg/ml [mode, 0.25 and 0.5 μg/ml; median, 0.5 μg/ml]) (Fig. 3a). The difference in the mean value for both data sets (Murray, 0.108 μg/ml; modern, 0.417 μg/ml) was statistically significant ($P < 0.01$). When MBC values were compared, the majority of modern isolates had values of 4 or >4 μg/ml (see Fig. S2a in the supplemental material). Three Murray isolates, M109, M295, and M296, also had MBC values of >4 μg/ml, but the majority were in the range of 0.06 to 0.25 μg/ml.

However, for skin antiseptics, i.e., chlorhexidine, benzylalkonium chloride (BAC), and hexadecylpyridinium chloride mono-

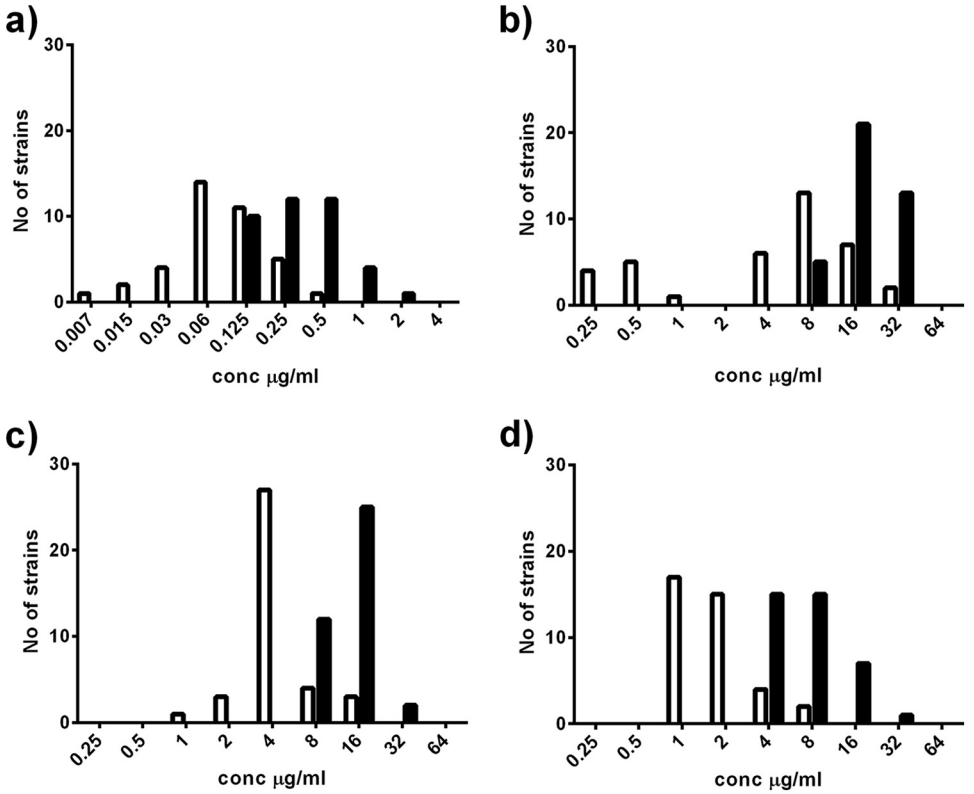

FIG 3 Susceptibility to clinical skin and wound disinfectants. MICs were determined for the Murray isolates (white bars) and the modern isolates (black bars) for triclosan (a) and the topical antiseptics chlorhexidine digluconate (b) and two quaternary ammonium cationic disinfectants, benzalkonium chloride (c) and HDPCM (d), and the number of strains plotted against their MIC levels.

hydrate (HDPCM), there was a wider susceptibility range in the Murray isolates for chlorhexidine than with the modern strains; MIC values for the Murray isolates were 0.25 to 32 μg/ml (mode, 8 μg/ml; median, 4 μg/ml), whereas in the modern strains the range was 8 to 32 μg/ml (mode, 16 μg/ml; median, 16 μg/ml) (Fig. 3b to d). For benzylalkonium chloride and HDPCM, there was a decrease in susceptibility in the modern isolates compared with the Murray isolates (for BAC, modes and medians of 4 and 16 μg/ml [Murray and modern isolates]; for HDPCM, modes of 1 and 8 μg/ml and medians of 3 and 12 μg/ml for the Murray and modern isolates, respectively). Similarly, although it was a smaller data set, when only the K1 capsular types were compared to reduce bias, then the range of susceptibilities was 0.5 to 16 (Murray) to 8 to 16 (modern) μg/ml for chlorhexidine, 2 to 8 (Murray) to 8 to 16 (modern) μg/ml for benzylalkonium chloride, and 1 to 4 (Murray) to 4 (modern) μg/ml for HDPCM. When the mean or median values were compared for chlorhexidine, benzylalkonium chloride, and HDPCM between the Murray and the modern data sets, the difference was found to be statistically significant ($P < 0.01$). MBC analysis on all these isolates showed a similar effect (see Fig. S2b to d in the supplemental material). To understand antiseptic resistance, the presence of the disinfectant resistance genes *cepA*, *qacE*, and *qac*Δ*E* was looked for in the Murray isolates. All were positive for the *cepA* gene, but no isolates were found to contain *qacE* or *qac*Δ*E*. Closer examination of the *cepA* sequences revealed that several isolates had either 9- or 18-bp insertions (see Table S1 in the supplemental material). Isolates that contained these insertions had lower MIC values (0.25 to 1 μg/ml) for chlorhexidine than the other Murray isolates without the insertion (4 to 32 μg/ml). All modern isolate *cepA* sequences also did not appear to contain insertions, but these isolates showed higher levels of chlorhexidine resistance. These insertions were also not detected in any published *Klebsiella cepA* sequences. For benzylalkonium chloride and HDPCM, again those isolates which contained insertions in *cepA* had higher levels of susceptibility, although, there were several non-insertion-containing isolates which had similar MIC levels.

DISCUSSION

The aim of this study was to analyze the traits of pre-antibiotic era *Klebsiella* isolates (Murray strains). There was a significant proportion of isolates that were resistant to all penicillins tested due to the presence of a bla_{SHV} gene. Resistance to penicillin was first described in 1942 in *Staphylococcus aureus*, and several of the Murray isolates predate this (22) (see Table S1 in the supplemental material). The bla_{SHV} genes were first described in *Klebsiella* as encoding chromosomally encoded narrow-spectrum β-lactamases (23, 24), but this gene (bla_{SHV-1}) and its subsequent narrow- and extended-spectrum variants have been found on plasmids in over 90% of modern *Klebsiella* clinical isolates (25).

Interestingly, one Murray isolate, M43, contained a full-length novel bla_{SHV} gene but did not show resistance to penicillins. When this gene was cloned into ampicillin-susceptible *E. coli*, it did not confer resistance. However, it was possible to generate mutants from this strain which were resistant to penicillins by passaging on medium containing ampicillin. These mutants included not

just amino acid substitutions (producing novel bla_{SHV} types) but also nucleotide changes in the promoter region around the ribosome binding site. Within clinical *E. coli* isolates, the level of another β-lactamase gene, bla_{TEM-1}, produced can vary by up to two orders of magnitude, resulting in a change in resistance (26, 27). Increased production of β-lactamase can also arise from mutations and insertions, which alter promoter strength, or gene duplications, e.g., having copies of β-lactamase genes on the chromosome and plasmids (28, 29).

The difference in the levels of susceptibility of the Murray and modern isolates to skin antiseptics and triclosan is intriguing. The disinfectants analyzed fell into two clear categories. There were no apparent differences with respect to their susceptibility to different oxidative disinfectants, whereas there were differences in the mean MIC/MBC values for the antiseptics and triclosan. Increased disinfectant resistance has previously been associated with mutations in existing genes, e.g., *fabI*, *fabV*, or *nfxB*, in triclosan resistance (30, 31) rather than, as for some antibiotics, acquisition of new genes. For example, modern staphylococcal isolates appear to be more resistant to triclosan than those isolated before the widespread introduction of triclosan (32). The role of certain efflux pumps in biocide susceptibility, such as *qacE*, *qacΔE*, and *cepA*, is inferred from analysis of gene carriage (33–35). The 9- or 18-bp insertion found in the *cepA* gene of certain Murray isolates implies that this gene is clearly important in chlorhexidine susceptibility, but whether this insertion causes inactivation of *cepA* or altered expression levels is unknown and will be further investigated. The presence of *qacE* or *qacΔE* was not detected in any of the Murray isolates, but the role of these genes is unclear since these genes were not found in the majority of the modern isolates tested. Indeed, a recent study on *Staphylococcus epidermidis* highlighted that carriage of *qacA* or *-B* genes did not lead to an increase in resistance to chlorhexidine (36).

There have currently been almost 1,800 different STs found in *Klebsiella* (bigsdb.web.pasteur.fr/klebsiella/klebsiella.html), and there are several important STs (e.g., ST23 and ST82) represented within the Murray collection which have been implicated in virulence (37). Modern *K. pneumoniae* strains, even when isolated from clinical cases, show a wide range of virulence phenotypes in *Galleria* (10, 38), but certain high-virulence clades, e.g., CC23, have been identified. The only Murray strain that showed virulence that was similar to the more virulent modern clinical isolates, M109 (ST23), is likely to be part of the CC23 complex due to the presence of the *allS* gene. This is also increased evidence that it is clonal type rather than capsular (K) type which is responsible for virulence. K1 capsular types have historically been thought to be more virulent, but the finding that the majority of Murray isolates of K1 capsular type showed little or no virulence in *Galleria* suggests that capsular type is not an important virulence factor in this model. Attributing *Klebsiella* virulence to specific genes is difficult, given the lack of detailed knowledge of *K. pneumoniae* virulence and the likelihood that this involves multiple factors affecting growth, attachment, and invasion of the host. There are also certain clades which are characterized by having particular putative virulence genes; e.g., in this study, all members of CC23K1 were positive for every virulence factor analyzed. This would likely be present in modern as well as older *Klebsiella* isolates. Other studies have shown that certain clades are characterized by the presence of particular virulence factors (17). Therefore, it is possible that with a proportion of Murray isolates being CC82K1, certain virulence genes will be under- or overrepresented. The fimbrial gene *mrkD* is an important gene in biofilm physiology and pathogenesis (39) and was shown here to have much greater representation in the modern strains, with a separate study demonstrating that it was present on the chromosomes of 95% of *Klebsiella* isolates tested ($n = 90$) (40). This highly diverse gene has begun to be isolated from plasmids in *E. coli* (40), and there are now distinct clades of this gene in *Klebsiella*. Introduction of *mrkD* variants, other putative virulence genes, or cosmid libraries into the naive background(s) provided by the Murray isolates would be a useful way of identifying novel virulence mechanisms.

It is possible that traits may have been lost from the Murray isolates during long-term storage; e.g., there may have been loss of virulence plasmids. Details of how the Murray collection was stored have been provided previously (41). The sigma factor RpoS is often used as a marker of the impact of long-term storage due to its role in survival, with mutations in *rpoS* associated with changes during storage (42, 43). We investigated the RpoS sequences for all Murray isolates and found them to be identical, with one exception, to those of modern strains, indicating that the impact of storage on the isolates is likely to be minimal. The exception, M109, had a premature stop codon leading to a truncated protein (data not shown). This did not appear to affect virulence or disinfectant susceptibility, since this strain had levels equivalent to those in the modern members of the CC23 complex.

While there is always a possibility that the Murray *K. pneumoniae* isolates are not exactly representative of *Klebsiella* isolates from this era, this study still provides an intriguing snapshot of pre-antibiotic era *Klebsiella* isolates. Further investigation will reveal important evolutionary aspects of antibiotic resistance acquisition and adaptation of strains to other modern environmental pressures, e.g., disinfection and bacterial competition.

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