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In vitro competition and cooperation in co-cultures of *Klebsiella pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus*

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ABSTRACT

Introduction: Polymicrobial infections contribute significantly to the burden of morbidity and mortality of infectious diseases. Because microbial interactions can be cooperative or competitive, this study sought to assess the in vitro interactions of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Materials and Methods: In vitro competition assays with *K. pneumoniae* (KP05), *P. aeruginosa* (ATCC27853) and *S. aureus* (ATCC25923) were performed in Luria Bertani (LB) broth at 37ºC under aerobic conditions. Duplex (1:1 ratios) and triplex culture combinations (1:1:1 ratio) contained 1 x 10^3 cells/ml of each bacterium. Cell numbers were estimated at 1-, 2-, 4-, 8-, 12-, 24- and 48-hr timepoints and generation times were determined alone or in combination with other bacteria. Competitive PCR was used to assess the detectability of selected virulence-associated genes (one from each bacterium) at 8-, 24- and 48- hr timepoints.

Results: While the growth curve for monocultures followed normal growth patterns, duplex and triplex co-cultures saw marked evidence of competition and cooperation between and among the bacteria. Of note, *S. aureus* grew better with *P. aeruginosa* versus co-culture with *K. pneumoniae*. *P. aeruginosa* did not produce any measurable growth in the presence of *S. aureus*. *K. pneumoniae* grew better with *S. aureus* and least with *P. aeruginosa*. Competitive PCR for organism-specific genes were in the order: *S. aureus* > *K. pneumoniae* > *P. aeruginosa* which mirrored the growth parameters observed.

Conclusion: The findings revealed that of the three bacteria, *S. aureus* was the dominant species in both duplex and tripartite cultures.

Keywords: Co-cultures; Competitive-PCR; Bacterial interactions; *K. pneumoniae*; *P. aeruginosa*; *S. aureus*

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INTRODUCTION

Polymicrobial infections, comprising more than one kind of microorganism, have become increasingly important public health issues. While these may include potential pathogens and commensals, which are communicating, competing or collaborating with each other (1), they often complicate treatment because of their individual potential for antimicrobial resistance and virulence. In many instances, the observed interactions are based on strain- and environmental-specific factors which work together to lead to poor patient prognosis.

Pseudomonas aeruginosa is responsible for severe hospital-acquired infections in the immunocompromised and chronic infections in cystic fibrosis patients. Its virulence depends on many cell-associated and extracellular factors, and include pili, exoenzyme S and other adhesins that allows adherence to epithelial cells. It also exhibits exotoxin A responsible of tissue necrosis, Phospholipase C, a thermo-labile haemolysin, elastase and alkaline protease (2).

Infections caused by *Staphylococcus aureus* represent the second most common encountered in the hospital setting and are usually severe (3,4). *S. aureus* is notorious for its vast array of pathogenic strategies and its ability to effect multiple disease sequalae (5).

Klebsiella pneumoniae, also considered an opportunistic pathogen, is responsible for life-threatening cases of pneumoniae, urinary tract infections, bloodstream infections and sepsis (6,7,8). There are four major virulence factors in *K. pneumoniae* namely, lipopolysaccharide (LPS), siderophores, capsule and fimbriae (pili).

P. aeruginosa and *S. aureus*, and to a lesser extent, *K. pneumoniae*, have been reported in polymicrobial infections, especially in antimicrobial resistant biofilms, and in acute and chronic non-healing wounds (9,10). The nature of co-existence of pathogens have been reported to include specific spatial organization in biofilms (11), protection against microbial and host-derived proteases (9), selective up-regulation and down-regulation of virulence-associated genes (12).

While mixed cultures have been examined for *P. aeruginosa* and *S. aureus*, and for *P. aeruginosa* and *K. pneumoniae*, there has been no report of a mixed culture involving all three pathogens. Consequently, this study sought to assess the in vitro interactions of *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*, especially in terms of growth parameters and competitive detection of select virulence-associated genes.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

Cultures of *Klebsiella pneumoniae* (KP05), *Pseudomonas aeruginosa* (ATCC 27853) and *Staphylococcus aureus* (ATCC25923) were grown in Luria Bertani broth (LB) broth at 37°C with shaking. *K. pneumoniae* KP05 was a multi-drug resistant, *mrkA*$^+$ clinical strain, which was classified as a good biofilm producer (13).

Growth curves and competition assays for *P. aeruginosa, K. pneumoniae* and *S. aureus* in co-cultures

Growth curves were done in LB at 37^{o}C under aerobic conditions with moderate shaking over a 48-hour period, and readings were taken at 1-, 2-, 4-, 8-, 12-, 24- and 48-hr timepoints. Co-cultures were inoculated with various combinations of pure cultures of *K. pneumoniae*, *P. aeruginosa* and *S. aureus* grown overnight (15 h, aerated, 37^{o}C, shaking), diluted to about 1 x 10^3 cells/ml, as follows: duplex culture combinations in a 1:1 ratio and triplex culture combinations in a

1:1:1 ratio. Bacteria were serially diluted in sterile phosphate-buffered saline (PBS), and plated in triplicates onto LB agar for pure cultures. Cell numbers for co-cultures were estimated on MacConkey agar (*K. pneumoniae* appear as pink translucent colonies), King B agar (selective media for *P. aeruginosa*) or Mannitol Salt Phenol Red Agar (selective media for pathogenic Staphylococci). The plates were incubated overnight at 37^{o}C and colony forming units (CFU/ml) determined. Significant differences were considered >2-fold and $p<0.05$. The generation time and the number of generations were estimated from the graph during the exponential phase in single, duplex and triplex cultures, based on the following equations:

K (growth rate) = ($\log B_n - \log B_0$)/log2×t ; G (generation time) = 1/K

where t = time interval in hours or minutes; B_0 = number of bacteria at the beginning of a time interval; B_n = number of bacteria at the end of the time interval

DNA isolation and Competitive PCR Analysis

Selected pathogen-associated genes: *shv* (β-lactamase) for *K. pneumoniae*, *toxA* (exotoxin A) for *P. aeruginosa*, and *hla* (alpha haemolysin) for *S. aureus*, were amplified by PCR at 8-, 24- and 48-hr timepoints. These genes were chosen because they were found more abundantly or solely in the specific bacterium and would provide a good surrogate or proxy for the organism's performance in the co-cultures. Total DNA was extracted according to the Promega Wizard Genomic DNA Purification Kit protocol and the DNA was stored at 2–8°C. Table 1 includes details of primer sequences for each target gene. The presence of each gene was screened by monoplex or multiplex, as appropriate, using the GeneAmp PCR System 9700 (Applied Biosystems, CA, USA) and the GoTaq Green Master mix (Promega Corp, Madison, WI, USA). The amplified products were separated using a 2% agarose gel in 0.5x TBE and visualized by staining with ethidium bromide and exposure to UV light via the 3UV Benchtop transilluminator.

RESULTS

Colonial growth and morphology

On LB media, *K. pneumoniae* produced large, opaque creamy-white raised mucoid colonies, *P. aeruginosa* as small, opaque, creamy-white, flat colonies, and *S. aureus* as medium, circular, creamy-brown, opaque flat colonies. When *K. pneumoniae* was grown in the presence of *S. aureus* or *P. aeruginosa* and plated on MacConkey agar, it produced large mucoid pink, spreading, convex colonies. While *P. aeruginosa* produced small creamy-white, flat colonies on King B agar when co-cultured with *K. pneumoniae*, it did not produce any measurable colonial growth when grown in the presence of *S. aureus*. *S. aureus* consistently produced medium yellow-brown circular opaque colony on Mannitol Salt agar irrespective of the bacterium with which it was co-cultured.

Growth curves for monoculture, duplex and tripartite culture for the three bacterial strains

Monocultures of *K. pneumoniae, P. aeruginosa* and *S. aureus* patterned a normal growth curve with a lag, log, stationary, and death phases (Fig.1). *S. aureus* and *P. aeruginosa* followed a similar pattern of growth and only showed separation after the 24 hr timepoint. *K. pneumoniae* had the steepest growth incline of the three bacteria. Growth rates and generation times for bacteria are as given in Table 2.

Table 1: Primer sequences for the specific genes used in this study

Target Gene	Primer	Nucleotide Sequence	Reference
shv	Forward Reverse	5'GGGTTATTCTTATTTGTCGC 3' 5' TTAGCGTTGCCAGTGCTC 3'	14
toxA	Forward Reverse	5' GGAGCGCAACTATCCCACT 3' 5' TGGTAGCCGACGAACACATA 3'	15
hla	Forward Reverse	5' CTGATTACTATCCAAGAAATTCGATTG 3' 5' CTTTCCAGCCTACTTTTTTATCAGT 3'	16

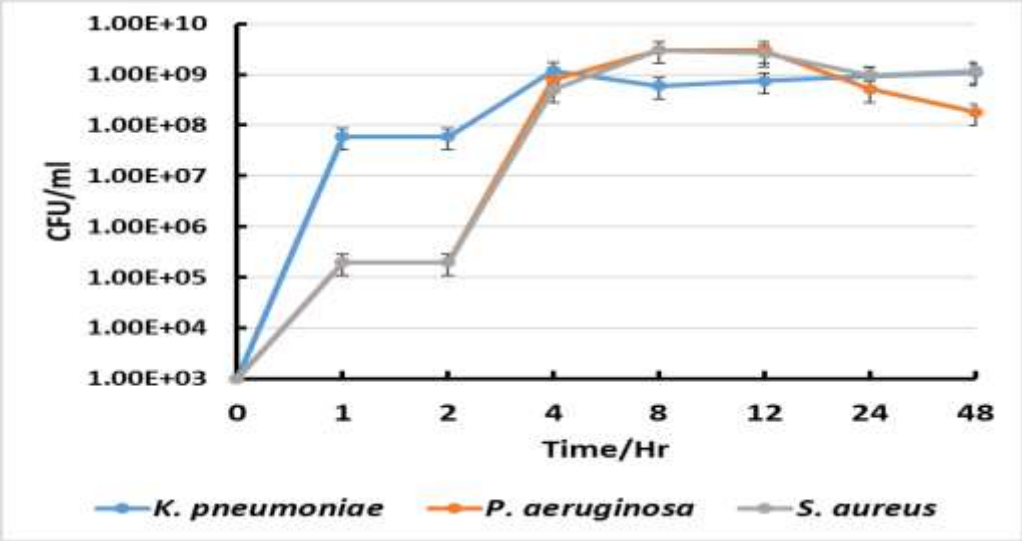

Figure 1- Growth curves of monocultures of *K. pneumoniae, P. aeruginosa and S. aureus*.

From the growth curve (Fig. 2.A), it was apparent that *K. pneumoniae*, although initially not achieving the higher cell density (CFU/ml) during the first hour when compared with *S. aureus*, performed best in terms of maintaining its presence in the co-culture mixtures. This was of the order of 1 x 10^8 CFU/ml after 48 hr and represented only a 1-log (i.e., 10-fold) reduction relative to the monoculture at the same timepoint.

In contrast, *P. aeruginosa* performed the worst of the three bacteria (Fig. 2.B). This was evident even though *P. aeruginosa* benefitted from a growth boost within the first hour of co-culture with *K. pneumoniae* and being able to maintain its cell density similar to the monoculture (1 x 10^8 CFU/ml), it produced no measurable growth in the presence of *S. aureus*. This observation was likely due to the presence of inhibitory substances secreted by *S aureus*, which was specific to *P. aeruginosa* and not *K. pneumoniae*.

S. aureus was intermediate with relatively high CFU/ml after 1 – 4 hr, but with a 2-log reduction in cell density in the presence of *K. pneumoniae* after 24 hr (Fig. 2.C). Interestingly, in the presence of *P. aeruginosa*, *S. aureus* numbers decreased 4-fold between 24 and 48 hr, but with *K. pneumoniae* present in a tripartite arrangement, the decrease in CFU/ml was even more significant: 2-log (i.e., 100-fold) at the 48-hr timepoint. This indicated that

whatever limited benefit *S. aureus* received from *P. aeruginosa* was eliminated in the presence of *K. pneumoniae*.

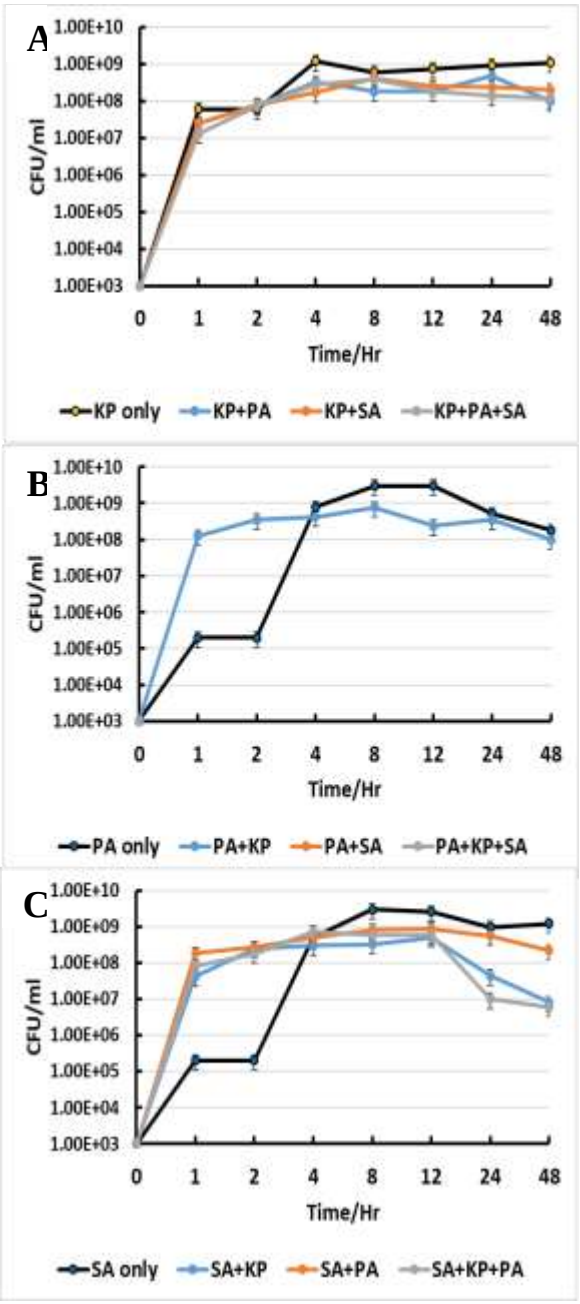

Figure 2. Composite growth curves showing the performance of (A) *K. pneumoniae* (KP) alone or co-cultured with *P. aeruginosa* and/or *S. aureus*; (B) *P. aeruginosa* (PA) alone or co-cultured with *K. pneumoniae* and/or *S. aureus*; and *S. aureus* (SA) alone or co-cultured with *K. pneumoniae* and/or *P. aeruginosa*

The generation times were calculated from the graph during the exponential phase of each bacterium and are shown in Table 2. In monoculture, *K. pneumoniae* had the lowest generation time of 26 minutes compared to the other two organisms. However, when co-cultured with *S. aureus* the generation time decreased by 10 minutes. Contrastingly, the generation time increased drastically in the presence of *P. aeruginosa*, by 33 minutes and in the triplex culture, *K. pneumoniae* had a generation time of 19 minutes, the lowest generation time for tripartite cultures. Overall, *K. pneumoniae*, grew rapidly when co-cultured with *S. aureus* than with *P. aeruginosa* and singly.

On the other hand, *P. aeruginosa* in monoculture had a generation time of 49 minutes. Interestingly, however, no growth was observed for *P. aeruginosa* in duplex culture with *S. aureus*, or in the tripartite culture, but it survived well when co-cultured with *K. pneumoniae* with a generation time of 31 minutes. Overall, *P. aeruginosa* grew better in duplex with *K. pneumoniae*, which had a difference of 18 minutes compared to growing singly. It was also vivid that *S. aureus* was inhibiting the growth of *P. aeruginosa*, but in presence of *K. pneumoniae* alone growth was enhanced.

Lastly, *S. aureus* had a generation time of 28 minutes, when co-cultured with *P. aeruginosa* it was lowered to 17 minutes but increased to 78 minutes with *K. pneumoniae.* Meanwhile, in tripartite culture the generation time was 54 minutes. Overall, *S. aureus* grew well when co- cultured with *P. aeruginosa*, which had a difference of 2 minutes for generation time when compared to growing singly.

Table 2. Generation time and growth rate for each bacterium during their exponential phase in single, duplex and triplex cultures.

Organisms in single, duplex and tripartite culture	Generation rate (min)	Generation time (min)
S. aureus	0.04	28
K. pneumoniae	0.04	26
P. aeruginosa	0.02	49
S. aureus with P. aeruginosa	0.06	17
S. aureus with K. pneumoniae	0.01	78
P. aeruginosa with S. aureus	0	0
P. aeruginosa with K. pneumoniae	0.03	31
K. pneumoniae with S. aureus	0.06	16
K. pneumoniae with. P. aeruginosa	0.01	59
S. aureus with K. pneumoniae & P. aeruginosa	0.02	54
K. pneumoniae with S. aureus & P. aeruginosa	0.05	19
P. aeruginosa with S. aureus & K. pneumoniae	0	0

Details of competitive PCR detection of organism-specific genes are given in Table 3. All genes were detected at the start of the experiment, and for the monocultures, genes were amplified at all-time points, except for *toxA* at 48 hr. The *shv* gene had some loss of signal at the 8 hr timepoint when *K. pneumoniae* was co-cultured with *P. aeruginosa* and further at the 24 and 48 hr timepoints. Decline in amplification signal strength was more pronounced in the tripartite combination from the 8-hr point onwards. For *toxA* detection, there was significant loss of signal at the 24 hr timepoint when *K. pneumoniae* was co-cultured with *P. aeruginosa* and abolished at the 48 hr timepoint. Abolition in signal was noted at the 24- and 48-hr timepoint with *S. aureus* and in the tripartite arrangement. For *hylA* detection, there was no loss of signal over the period of the experiment and was the case when *S. aureus* was cultured alone or in combination with other bacteria. The observations were largely in line with the growth parameters observed.

Table 3. Detection of organism-specific genes by competitive PCR

	Incubation time (hr)			
Organism (gene)	**0**	**8**	**24**	**48**
KP (*shv*)	+++	+++	+++	+++
+ PA	+++	++	+	+
+ SA	+++	+++	+++	+++
+ PA + SA	+++	+	+	+
PA (*toxA*)	+++	+++	+++	0
+ KP	+++	+++	+	0
+ SA	+++	+++	0	0
+ KP + SA	+++	+++	0	0
SA (*hylA*)	+++	+++	+++	+++
+ KP	+++	+++	+++	+++
+ PA	+++	+++	+++	+++
+ KP + PA	+++	+++	+++	+++

Key: KP = *K. pneumoniae*; PA = *P. aeruginosa*; SA = *S. aureus* +++/++/+, present; 0, not detected

DISCUSSION

In this study, the in vitro interactions of *K. pneumoniae*, *P. aeruginosa* and *S. aureus* indicated that bacteria compete and cooperate in co-cultures. Despite, its array of virulence factors and usual aggressive growth parameters, *P. aeruginosa* found the going tough in the presence of *S. aureus*. Foremost, the acquisition of nutrients is crucial in bacterial competition and it must be noted that *S. aureus* reproduced more rapidly than *P. aeruginosa* and this could be a pillar for the absence of detectable growth of *P. aeruginosa*. As they both have the same nutritional requirements, the bacterium with the faster growth rate would be more competitive, and hence favour the growing population of *S. aureus*. However, it is well-established that *S. aureus*, as a ubiquitous commensal on the skin and nasal passage, and with its own vast array of toxigenic and enzymatic virulence factors (many of which are either cell-associated or secreted) can effectively establish itself in different niches. These factors, as well as its propensity for aggressive behaviour, could have led to the inhibition of *P. aeruginosa* (17).

Other studies have opposing views of the competitiveness of *P. aeruginosa*. For instance, the study by Goldsworthy *et al.*, (18), revealed that when *P. aeruginosa* and methicillin-resistant *S. aureus*, both common in catheter-associated urinary tract infections (CAUTI), were grown together as a mixed culture within a CAUTI model, *P. aeruginosa* produced accelerated biofilm in comparison to monocultures. In that study, the authors used real-time quantitative PCR to demonstrate that exotoxin A production by *P. aeruginosa* was increased 1839-fold in the mixed culture, while not showing any significant gene expression of α-haemolysin in MRSA in either culture.

Unlike the antagonistic relationship between *P. aeruginosa* and *S. aureus*, *K. pneumonia* grew in the presence of both, especially in combination with *S. aureus*. In fact, the generation time for *K. pneumoniae* was lowest when co-cultured with *S. aureus*, meaning that it produced colonies even faster than growing singly or in combination with *P. aeruginosa*.

S. aureus and *K. pneumoniae* are both encapsulated and therefore this protective outer layer could have played a vital role in their survival together, while competing for space and protection from the release of toxic or other virulence/inhibitory factors. While some studies report that bacteria can co-exist, many are limited to intra-species co-evolution, and very few have examined the potential for co-evolution in mixed populations. However, Hibbing *et al.*, (1) demonstrated commensal interaction and co-evolution of *P. putida* and *Acinetobacter* sp. In that study, the authors showed that *P. putida* depended on the partner organism *Acinetobacter* sp. strain C6 to grow on benzyl alcohol as a sole carbon source, thus leading to greater overall growth yield in the biofilm co-cultures. This was despite the *P. putida* having a detrimental effect on the growth of *Acinetobacter*. Hibbing, et al., (1) also noted that competing microbes could stably coexist under certain nutrient concentration ratios, as seen in this study with *S. aureus* and *K. pneumoniae* and *P. aeruginosa* with *K. pneumoniae*.

Contrastingly, in other conditions, specific taxa can be outcompeted due to acute nutrient limitation as seen in co-cultures of *S. aureus* with *P. aeruginosa.* The overall significance of this study demonstrated interspecies competition and co-evolution does exist. Mixed cultures can exist stably while at the same time one can suppress the growth of the other. Thus, it very important to understand the relationship between different bacterial species, has it can identify the most competitive and dominant species in a mixed infection. This depends on the fast acquisition of resources, rapid generation

time, genetic malleability and other engaging activities which contribute or influences the succession of bacteria in competition (1).

In vitro studies have several limitations. One such is the difficulty in linking competitive behaviour and the predictions generated from in vitro studies, since they have not been tested in more natural settings. For example, our results might have differed from that of Goldsworthy *et al*., (18) since they used very specific modelling of an infection. Clearly, the use of a model (including lab animals) might better represent the natural environment, when compared to in vitro analyses. Another limitation to this study, was the selection of one gene from each bacterium for the competitive PCR analysis. In cases where a gene was not detected by PCR, this might have been due to possible changes in cell wall parameters which affected efficiency of lysis; via inhibition of the specific amplification by a component produced by the competitor strain; or due to death or loss of viability of the organism which resulted in significant reduction in overall available template. While the competitive PCR mirrored closely the phenotypic results of growth in co-cultures, quantitative real-time PCR would be more effective in indicating any changes in competition-based gene expression.

CONCLUSION

Taken together, this study showed that co-cultures of *K. pneumoniae*, *P. aeruginosa* and *S. aureus* can result in aggressive competition or peaceful co-existence. Clearly, knowledge of bacterial interactions and likely dominant species in a mixed infection will be valuable for effective treatment especially in serious nosocomial cases. In this study, *S. aureus* was the most dominant and competitive species of the three bacteria analysed.

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