In vivo correlates of molecularly inferred virulence among extraintestinal pathogenic Escherichia coli (ExPEC) in the wax moth Galleria mellonella model system

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In contrast to commensal Escherichia coli, extraintestinal pathogenic E. coli (ExPEC) strains possess an array of virulence-associated genes. We sought to establish the feasibility of using the invertebrate Galleria mellonella (greater wax moth) for assessing ExPEC virulence and to investigate the correlation between genotypic determinants of virulence and in vivo pathogenicity. We observed a correlation between the number of virulence genes and larval survival, such that ExPEC isolates with higher virulence scores killed larvae significantly faster than isolates with lower virulence scores. By correlating genotypic and phenotypic virulence, we provide preliminary validation of this model for future studies investigating ExPEC virulence.

Introduction

Escherichia coli is a major human pathogen, and infections caused by E. coli are associated with significant morbidity and mortality.1 Human-associated strains of E. coli can be broadly classified into three main pathotypes: (1) commensal E. coli strains, which exist as part of the normal gastrointestinal flora, (2) intestinal pathogenic E. coli strains, such as enterohemorrhagic E. coli (EHEC) and enteropathogenic E. coli (EPEC), and (3) extraintestinal pathogenic E. coli (ExPEC).2 Although most commonly associated with uncomplicated urinary tract infections, ExPEC strains cause a range of serious infections, including meningitis, pneumonia, and bacteremia.1

In contrast to commensal E. coli, ExPEC strains possess an array of virulence-associated genes including those encoding adhesins, invasins, siderophores, and polysaccharide capsules.3,4 These putative virulence factors, often encoded on mobile pathogenicity islands, enable ExPEC strains to successfully colonize, and in some instances invade, their human hosts. However, despite considerable characterization of the molecular epidemiology and virulence gene repertoire of ExPEC strains,5-7 studies assessing in vivo correlates of molecular virulence potential are less common. Such studies are important, not only for assessing previously identified virulence traits, but also for identifying new candidate virulence factors and potential therapeutic targets.

Recently, larvae of the greater wax moth Galleria mellonella have been increasingly utilized as an alternative model host to study microbial pathogenesis.8 Previous studies have demonstrated the utility of this simple invertebrate model system in studying the virulence of several major human extraintestinal pathogens, including Candida albicans,9 Acinetobacter baumannii,9 Staphylococcus aureus,10 and Pseudomonas aeruginosa.11 Importantly, for some pathogens, virulence in G. mellonella has been shown to correlate with both molecular virulence characteristics and pathogenicity in mammalian model systems.12 To date however, no studies have assessed the efficacy of the G. mellonella model for studies of ExPEC pathogenicity, although one previous study demonstrated the utility of this model for studying EPEC virulence.13 Accordingly, we sought to: (1) to establish the feasibility and reproducibility of using G. mellonella as a host for assessing ExPEC virulence and (2) to investigate the comparative virulence of a collection of molecularly characterized ExPEC strains, with particular regard to correlation of molecularly inferred virulence and experimental in vivo pathogenicity.

Results and Discussion

Infection characteristics of E. coli strains in G. mellonella
To investigate the feasibility of using G. mellonella to study E. coli pathogenicity, we evaluated larval survival using increasing
inocula of an E. coli reference strain (ATCC 25922). We observed a dose-dependent response on larval survival, such that increasing concentrations of bacterial cells killed larvae significantly faster (Fig. 1). At doses below 5 x 10^6 CFU, less than 30% of larvae were killed after 4 d. In contrast, at doses above 5 x 10^6 CFU, over 80% of larvae were killed within 24 h (P < 0.001). A dose of 1 x 10^6 CFU consistently killed 30–40% of larvae by 72 h (Fig. 1), and was therefore considered to provide enough discriminatory power to determine comparative differences in ExPEC virulence in subsequent experiments.

In order to determine whether viable E. coli cells were required for larval killing, we inoculated larvae with heat-killed E. coli ATCC 25922 or the heat-killed clinical isolate UOAE8433. When followed for 125 h post-injection, no deaths occurred among larvae inoculated with either PBS or heat-killed E. coli (data not shown). We also assessed whether larval killing may be due to E. coli secretory products rather than live bacterial cells. However, when larvae were inoculated with filter-sterilized bacterial supernatants taken from stationary-phase growth cultures, no larval killing was observed after 125 h (data not shown). In keeping with other studies of bacterial pathogenesis in this model, these results indicate that viable E. coli cells are required for killing of G. mellonella.14 Future work should attempt to elucidate the specific mechanism(s) of larval death as a result of E. coli infection.

We also determined the effect of administration of an antimicrobial agent (ciprofloxacin) on survival of G. mellonella infected with clinical isolates previously determined to be either susceptible or resistant to ciprofloxacin in vitro. We found that among larvae inoculated with a ciprofloxacin-susceptible isolate (UOAE8502), the ciprofloxacin-treated group had significantly improved survival at 4 d compared with the non-treated group (90% vs. 35%; P < 0.001) (Fig. 2). In contrast, when larvae were inoculated with a ciprofloxacin-resistant isolate (UOAE8433), ciprofloxacin did not improve larval survival, with no significant difference between the treated and non-treated groups (P = 0.97).

Therefore, our data document a correlation of in vitro antimicrobial resistance with in vivo response in this model system, which suggest that G. mellonella may be a useful model for future studies assessing antimicrobial efficacy against ExPEC strains prior to mammalian experimentation. For example, screening of novel agents in G. mellonella could conceivably rapidly identify those that have poor in vivo efficacy, thereby reducing the extent of subsequent mammalian testing required.

In vivo correlates of genotypic virulence determinants in G. mellonella

For invertebrate hosts such as G. mellonella to be useful extrapolative models for studies of in vivo pathogenicity, a correlation between genotypic determinants of virulence (as identified through human epidemiological studies and animal experimental studies) and phenotypic virulence (i.e., larval death) should exist. We therefore sought to use the G. mellonella model to assess the comparative virulence of a collection of clinical E. coli isolates that had previously been characterized for a broad range of virulence-associated genes, then to compare larval killing with virulence genotype.15 Clinical and molecular characteristics of study strains are shown in Tables 1 and 2. The aggregate virulence gene scores of the clinical isolates ranged from 4 to 23.

After 125 h, no deaths were observed in larvae inoculated with PBS, or the reference strains MG1655 or DH10b (data not shown). However, we observed a significant association between the aggregate virulence gene score and larval killing at 125 h (R^2 = 0.54, P = 0.02), such that ExPEC isolates with higher scores killed larvae significantly faster and in greater numbers than isolates with lower scores (Fig. 3). For example, ExPEC isolates (with their aggregate virulence scores listed parenthetically) UOAE6384 (13), UOAE8433 (19), and UOAE8502 (23) killed >65% of larvae at 125 h, whereas isolates UOAE3153 (4), UOAE1656 (7), and UOAE4027 (10) killed <10% of larvae (Fig. 3). This is in keeping with the association previously observed between molecularly inferred virulence score and lethality in a murine model.16
Virulence scores were calculated based on the number of virulence-associated genes detected after adjusting for multiple detection of the following operons: pap, kps (group 2 capsule), sfa/foc (P fimbriae), and sfa/foc (S and F1C fimbriae).

The lack of larval deaths after inoculation with isolates UOAE3153 (4), UOAE1656 (7), and UOAE4027 (10a) suggests that the virulence genes possessed by these isolates are either not expressed during infection of G. mellonella larvae or, if expressed, do not significantly diminish larval survival. Further research investigating ExPEC virulence in this model could include mutagenesis or knockout studies assessing putative ExPEC virulence factors. Interestingly, strain UOAE0886, which had an aggregate virulence gene score of 11, demonstrated the fastest larval killing. It is therefore possible that virulence genes other than those assessed by the multiplex PCR assay we used play an important role in larval killing. In addition, the level and timing of gene expression, and presence of minor genotypic polymorphisms may also significantly influence the effect of certain genes on pathogenicity.

**Conclusions**

To our knowledge, this represents the first study to assess the utility of the wax moth G. mellonella as a model host for studying ExPEC pathogenicity and virulence. We found that larval killing was: (1) dependent on the challenge E. coli strain being alive, (2) different among different ExPEC strains, (3) prevented by administration of an appropriate antimicrobial, and (4) significantly associated with aggregate virulence gene score. By correlating genotypic and phenotypic virulence in this way, our proof-of-principle study provides preliminary validation of this simple, non-mammalian model for further investigation of the virulence of this major human pathogen. Future studies should correlate these findings with observed virulence in mammalian systems.

**Methods**

**Strains and molecular characterization**

The E. coli isolates used in this study are shown in Table 1. Clinical isolates were from patients with E. coli bloodstream infections; these isolates were obtained from a previous study performed in the Department of Clinical Microbiology, Auckland City Hospital, New Zealand. Identification and antimicrobial susceptibility testing of these isolates was done as part of this prior study. Two K12-derived E. coli laboratory reference strains, MG1655 and DH10b, were used for comparison in larval survival experiments. In addition, an E. coli reference strain commonly used in antibiotic susceptibility testing (ATCC 25922) was used to establish the appropriate inocula for subsequent experiments and to determine whether viable E. coli cells were required for larval killing. Prior to use, all strains were cultured on Luria–Bertani (LB) agar or in LB broth and incubated aerobically at 37 °C. Growth kinetic curves were performed with all strains prior to use in the G. mellonella assay.

Molecular characterization of E. coli isolates was performed using previously described methods. Briefly, a multiplex PCR was used to detect 50 virulence-associated genes and their allelic variants, and an aggregate virulence gene score was assigned to each isolate as previously described. Depending on the number of virulence-associated genes detected, after adjusting for multiple detection of the following operons: pap (P fimbriae), sfa/foc (S and F1C fimbriae), and kps (group 2 capsule).

**Galleria mellonella infection model**

G. mellonella larvae in the fifth instar larval phase (Biosuppliers Ltd) were stored in the dark at room temperature and were used within seven days of receipt. All larvae were between 100 and 200 mg and displayed minimal melanization prior to use. For each experiment, larvae were infected by inoculating 20 μL of the prepared inoculum into the hemocoel through the last left proleg using an insulin syringe. Before inoculation, bacterial cells were washed in phosphate buffered saline (PBS) and re-suspended to give the appropriate inocula, which were confirmed by retrospective plating onto LB agar. All experimental groups contained 10 randomly selected larvae and each experiment was repeated at least three times using larvae from different batches. In all experiments, PBS was used as an

| Strain     | Year of isolation | Source of strain                  | Virulence score |
|------------|-------------------|-----------------------------------|-----------------|
| UOAE3153   | 2007              | Bacteremia post-prostate biopsy    | 4               |
| UOAE8433   | 2007              | Bacteremia following urosepsis     | 19              |
| UOAE8502   | 2008              | Bacteremia related to urosepsis    | 23              |
| UOAE0886   | 2008              | Bacteremia post-prostate biopsy    | 11              |
| UOAE0212   | 2009              | Bacteremia post-prostate biopsy    | 11              |
| UOAE6392   | 2009              | Bacteremia post-prostate biopsy    | 10              |
| UOAE4027   | 2009              | Bacteremia post-prostate biopsy    | 10              |
| UOAE1656   | 2009              | Bacteremia related to urosepsis    | 7               |
| UOAE6384   | 2010              | Bacteremia post-prostate biopsy    | 13              |
| MG1655     | -                 | Reference strain                   | 3               |
| DH10b      | -                 | Reference strain                   | 4               |

Virulence scores were calculated based on the number of virulence-associated genes detected after adjusting for multiple detection of the following operons: pap (P fimbriae), sfa/foc (S and F1C fimbriae), and kps (group 2 capsule).
Table 2. Genotypic virulence determinants of *Escherichia coli* strains used in *Galleria mellonella* survival experiments as determined by multiplex PCR.

| Functional category | Virulence gene(s) a, b | Strain | MG1655 | DH10b | UOAE1153 (V4) | UOAE1656 (V7) | UOAE4027 (V10a) | UOAE6392 (V10b) | UOAE0886 (V11b) | UOAE0212 (V11a) | UOAE6384 (V13) | UOAE8433 (V19) | UOAE8502 (V23) |
|---------------------|------------------------|--------|--------|--------|--------------|--------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
| **Adhesins**        | *papACEFG*             |        | -      | -      | -             | -             | -              | +              | -              | -              | +              | +              | -              |
|                     | *fimH*                 |        | +      | -      | +             | +             | -              | +              | +              | +              | +              | +              | -              |
|                     | *sfa/focDE*            |        | -      | +      | +             | -             | -              | +              | -              | +              | +              | +              | +              |
|                     | *iha*                  |        | -      | -      | -             | -             | -              | +              | +              | -              | +              | +              | +              |
|                     | *hre*                  |        | -      | -      | -             | -             | -              | -              | -              | -              | +              | +              | -              |
| **Capsule-**        | **associated**         |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *kpsMII*               |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *kpsMIII*              |        | -      | -      | -             | -             | -              | -              | -              | -              | -              | -              | -              |
| **Toxins**          | *hlyA*                 |        | -      | -      | -             | -             | -              | -              | -              | -              | -              | -              | -              |
|                     | *sat*                  |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | -              |
|                     | *vat*                  |        | -      | -      | -             | -             | -              | -              | +              | +              | +              | +              | +              |
|                     | *crfI*                 |        | -      | -      | -             | -             | -              | -              | -              | -              | +              | +              | +              |
|                     | *cdtB*                 |        | -      | -      | -             | -             | -              | -              | -              | -              | -              | -              | -              |
| **Protectins and**  | **invasins**           |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *traT*                 |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *iss*                  |        | -      | +      | -             | -             | -              | -              | -              | -              | -              | -              | -              |
|                     | *ibaE*                 |        | -      | -      | +             | -             | -              | -              | -              | -              | -              | -              | -              |
| **Siderophores**    |                       |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *iutA*                 |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *fyuA*                 |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *iroN*                 |        | -      | -      | -             | -             | -              | -              | -              | -              | +              | +              | +              |
|                     | *ireA*                 |        | -      | -      | -             | -             | -              | -              | -              | -              | +              | +              | +              |
| **Miscellaneous**   |                       |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *usp*                  |        | -      | -      | +             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *uidA*                 |        | +      | +      | +             | +             | +              | +              | +              | +              | +              | +              | +              |
|                     | *malX*                 |        | -      | -      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *clbB*                 |        | -      | -      | -             | -             | -              | -              | -              | -              | -              | -              | -              |
|                     | *ompT*                 |        | +      | +      | -             | -             | -              | +              | +              | +              | +              | +              | +              |
|                     | *pic*                  |        | -      | -      | -             | -             | -              | -              | -              | -              | -              | -              | +              |

Key: -, absent; +, present. *Gene definitions:* *cdtB*, cytolethal distending toxin; *clb* and *clbN*, colibactin synthesis; *cnf1*, cytotoxin necrotizing factor 1; *fimH*, type 1 fimbriae; *fyuA*, yersiniabactin (siderophore) receptor; *hlyA*, α-hemolysin; *hre*, heat-resistant agglutinin; *ibaE*, invasion of brain endothelium; *iha*, adhesin-siderophore; *ireA*, siderophore receptor; *iroN*, salmochelin (siderophore) receptor; *iss*, increased serum survival; *iutA*, aerobactin (siderophore) receptor; *kpsMII*, group 2 capsule; *kpsMIII*, group 3 capsule; *malX*, pathogenicity island marker; *ompT*, outer membrane protease T; *pic*, serine protease; *sat*, secreted autotransporter toxin; *sfa/focDE*, S or F1C fimbriae; *traT*, serum resistance-associated; *usp*, uropathogenic-specific protein; *uidA*, β-glucoronidase; *vat*, vacuolating toxin. aAll isolates tested negative for the following genes: *afaE8*, afimbrial adhesin; *bmaE*, M fimbriae; *gafD*, G fimbriae; *afa/draBC*, Dr-binding adhesins; *tsh*, temperature-sensitive hemagglutinin; *hlyF*, hemolysin; *easT*, heat-stable toxin; *rfc*, 04 lipopolysaccharide synthesis; *cvrC*, colicin; *H7*, flagellar antigen H7. b*Genes of the P fimbriae operon, although detected separately, yielded similar results, and are listed as one virulence trait: *papA*, P fimbrial structural subunit; *papC*, assembly; *papEF*, tip pilins; *papG*, adhesin.

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inoculation control. Post-inoculation, larvae were housed without food in 24-well plates and incubated at 37 °C. Dead larvae were enumerated daily. Larvae were considered dead when they failed to respond to touch.

To determine whether viable E. coli cells were required for larval killing, heat-killed E. coli strains (ATCC 25922 and one of the clinical strains: UOAE8433 [Table 1]) were used to infect larvae. Briefly, cells were heated at 80 °C for 30 min and plated on LB agar to ensure that no viable cells were remaining. In addition, larvae were also inoculated with filter-sterilized supernatant obtained from these two strains in stationary phase growth cultures to assess the possible effect of bacterial secretory products on larval survival.

For antimicrobial efficacy testing, groups of larvae were inoculated with a lethal inoculum of E. coli clinical isolates that had been previously determined to be either susceptible (UOAE8502) or resistant to ciprofloxacin (UOAE8433) in vitro. Within 30 min of inoculation, ciprofloxacin was administered by injection into a different proleg. The PBS control group was injected twice, to control for the dual administration of both inoculum and antibiotic. Ciprofloxacin was administered at 10 mg kg⁻¹ of body weight, based on human dosing criteria.

Statistical analysis
Survival curves were generated using the Kaplan–Meier method, while the degree of association between the virulence gene score and larval survival was determined using the Pearson correlation coefficient. All statistical analysis was performed using GraphPad Prism (Version 6.0). A P value of < 0.05 was considered to indicate a statistically significant difference.

Disclosure of Potential Conflicts of Interests
J.R.J. has received grants or contracts from Merck, Rochester Medical, and Syntiron, and has patent applications regarding molecular tests for E. coli clonal groups. All other authors have no conflicts to report.

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