Development of a novel ex vivo insect model for studying virulence determinants of *Escherichia coli* K1

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A key step in *Escherichia coli* K1 meningitis is the crossing of the blood-brain barrier by the bacteria in order to gain entry into the central nervous system (CNS). In this study, a novel ex vivo model to study *E. coli* K1 invasion of the CNS is described that uses the African migratory locust, *Locusta migratoria*. By injecting bacteria into isolated locust head capsules, it was demonstrated that *E. coli* K1 invade the locust brain within 2 h in numbers depending on the concentration of bacteria injected. Using several mutants derived from K1, it was shown that outer-membrane protein A is a critical bacterial determinant required for the *E. coli* K1 invasion. The isogenic gene-deletion mutants, *ΔfimH, Δcnf1, ΔneuDB* and a rough LPS mutant showed significantly reduced invasion of locust brain. This novel model for the study of *E. coli* K1 pathogenesis offers several advantages over existing mammalian models in relation to its relative ease of use, cost-effectiveness and ethical acceptability.

INTRODUCTION

Neonatal bacterial meningitis caused by *Escherichia coli* (more than 80% due to *E. coli* strains possessing K1 antigen) contributes to more than 50,000 annual deaths worldwide, and it is of serious concern that these numbers remain significant, despite advances in antimicrobial chemotherapy (de Louvois et al., 1991; Unhanand et al., 1993; Kim, 2002, 2006). Although haematogenous spread is known to be a pre-requisite in *E. coli* meningitis, it is not clear how circulating bacteria cross the blood-brain barrier to gain entry into the central nervous system (CNS) to produce disease (reviewed by Kim, 2001, 2002, 2006). Over the last few decades only a handful of bacterial virulence determinants (such as outer-membrane protein A, FimH, cytotoxic necrotizing factor-1, Ibe proteins, Traj and AsLA) have been identified as associated with *E. coli* K1 pathogenesis (Kim, 2006). The *E. coli* genome possesses approximately 5.3 Mbp, and approximately 500 kb of the *E. coli* K1 genome (RS218 strain) is not present in the genome of the *E. coli* K-12 strain MG1655, so it is reasonable to anticipate the existence of several as yet unknown virulence determinants involved in *E. coli* meningitis. Current studies have relied upon vertebrate models to study *E. coli* pathogenesis, but mammalian models are expensive, not routinely available in many laboratories, require labour-intensive management and have ethical implications. There are many parallels between the innate immune responses of mammals and insects, and it is envisaged that insects could make useful models for the study of disease pathogenesis (Scully & Bidochka, 2006), especially as insects possess a highly selective blood-brain barrier exhibiting functional properties comparable with that of vertebrates (Carlson et al., 2000). Here, *Locusta migratoria*, the African migratory locust, is used as a model to study *E. coli* pathogenesis.

The aim of the present study was to develop an *ex vivo* locust model specifically to study invasion of the CNS by *E. coli*. To achieve this, ligated head capsules were isolated and injected with high concentrations of bacteria. In this way, the ability of the locust immune system to respond to infection was severely impaired, producing an ideal environment where the progress of bacteria crossing the blood-brain barrier could be studied. Thus, by using various mutants derived from K1 strain RS218, it was possible to test in the locust the importance of several factors thought to be important for CNS invasion in mammals.

METHODS

**Locusts.** African migratory locusts, *Locusta migratoria* were bred routinely at Birkbeck College; they were fed on bran, wheat seedlings and fresh grass as described previously (Goldsworthy et al., 2003). Adult male locusts used in the experiments described here were 15–30 days old.
**RESULTS AND DISCUSSION**

**E. coli K1 but not K12 invaded locust brain resulting in death**

To determine whether *E. coli* kill locusts, mortality assays were performed. The findings revealed that all locusts infected with K1 died within 72 h, while K12-infected locusts showed less than 5% mortality for up to 10 days. One possible explanation of these differences between K1 and K12 could be that K1 survives and multiplies within the haemolymph of the locust, eventually invading the CNS, whereas K12 does not. To test this hypothesis, locusts were injected with K1 or K12 and after 24 h, brains were dissected out, treated with gentamicin and intracellular bacterial counts were determined as described above. Of note, the PBS post-gentamicin wash plated on nutrient agar plate did not yield any bacterial c.f.u., confirming that the antibiotic treatment was effective. Brain lysates of infected locusts with K1 showed bacterial counts of approximately 10 000 ± 560 c.f.u. per brain, while K12-infected locusts did not yield any bacterial c.f.u.

**E. coli K1 invaded the locust brain in a concentration-dependent manner**

To determine the optimal numbers of *E. coli* K1 to be injected, isolated head capsules were injected with varying numbers of bacteria and incubated for 2 h before removal of the brains, washing, lysing and plating. The results revealed that *E. coli* K1 invaded the locust brain in a concentration-dependent manner up to 5 × 10^5 c.f.u. injected per head (Table 1). The results obtained with the initial inoculum of 10 000 c.f.u. were significantly different from all other values except with the highest bacterial inoculum of 10 000 000 (*P* = 0.023 for 50 000 bacterial inoculum, *P* = 0.013 for 100 000, *P* = 0.009 for 400 000, *P* = 0.0099 for 500 000, using paired *t*-test, one-tailed distribution). Although small numbers of bacteria were recovered from the brains taken from head capsules injected with as few as 1 × 10^2 *E. coli* K1, the optimal number of injected bacteria for the purposes of the assay was taken as 5 × 10^5. At this level of injected bacteria, about 0.1% of the original inoculum was recovered from a single brain within the 2 h incubation period (Table 1). Injections of higher numbers of bacteria reduced their recovery from injected with numbers of bacteria ranging from 10^4 – 10^6. Injections were performed using an automatic pipette in which the plastic tip was modified by the insertion of a short length of stainless steel hypodermic needle held in the bore by friction. In this way, small volumes of bacterial suspension (usually 5 μl) could be injected into the locust’s head capsule by inserting the needle into the ‘cheek’ (the gena) on one side of the head just behind the subocular sulcus and 2–3 mm below the compound eye. Care was taken to inject just below the surface of the cuticle so that there was no possibility of injecting directly into the foregut or the brain. The injected head capsules were incubated for various intervals at 37 °C. At the end of the incubation, the brain was dissected out as described above.

**Preparation of isolated head capsules from locusts.** Locusts were ligated in the neck region using a loop of fine thread (Ameto gena) on one side of the head just behind the subocular sulcus and 2–3 mm below the compound eye. Care was taken to inject just below the surface of the cuticle so that there was no possibility of injecting directly into the foregut or the brain. The injected head capsules were incubated for various intervals at 37 °C. At the end of the incubation, the brain was dissected out as described above.

**Insect model for studying *E. coli* virulence determinants.** *E. coli* K1 strain RS218 (O18:K1:H7) is a cerebrospinal fluid isolate from a neonate with meningitis (Silver et al., 1980; Achtmann et al., 1983). Several isogenic gene-deletion mutants of K1 were used, including a fimH-deletion mutant (*α* fimH) (Alsam et al., 2006; Khan et al., 2007), an ompA-deletion mutant (*α* ompA) (Khan et al., 2003) and a cnf1-deletion mutant (*α* cnf1) (Khan et al., 2002, 2003). In addition, a capsule-deletion mutant (*α* neuD) derived from K1 was used (Kim et al., 2003; Jung et al., 2007). This mutant lacks the neuD gene cluster that is necessary for the production of cytoplasmic precursors to the exopolysaccharide capsule. A rough LPS mutant, constructed using chemical mutagenesis, was also used. For simplicity, the rough LPS mutant is referred to as ΔLPS, although there were no genetic manipulations (Kim et al., 1992). *E. coli* K12 strain HB101, a non-invasive isolate was used as a negative control. For simplicity, *E. coli* K1 strain RS218 is referred to as K1, while *E. coli* K12 strain HB101 is referred to as K12 throughout the manuscript. Bacterial strains were routinely grown at 37 °C in Luria–Bertani broth (LB) and bacterial c.f.u. determined by plating on nutrient agar plates. Where appropriate to the strain being cultured, the medium was supplemented with ampicillin (100 μg ml⁻¹), kanamycin (40 μg ml⁻¹), chloramphenicol (25 μg ml⁻¹) or rifampicin (50 μg ml⁻¹).

**Mortality assays.** To determine whether *E. coli* kill locusts, K1 and K12 were injected into locusts (60 locusts per strain). Each locust was injected with 2 × 10⁶ c.f.u. *E. coli* suspended in 20 μl LB broth into the haemocoel of the locust abdomen by inserting the needle into the intersegmental membrane between two abdominal terga. Locusts were kept in plastic ‘critter cages’ and mortality was recorded every 24 h.

**CNS invasion assays in vivo.** To determine *E. coli* association with the CNS, locusts were injected with bacteria as above. The brains were dissected from each locust by making a sagittal cut through the base of the left antenna, removing the left side of the head, and the brain dissected out from the remaining right hand side using fine forceps after severing the circumoesophageal connectives and the optic tract to the right compound eye. Care was taken to ensure that both cerebral ganglia were present and that they were free of fat body tissue and air sacs. The dissecting instruments were surface-sterilized with methylated spirit between dissections. Each brain was placed separately in a 1 ml plastic centrifuge tube containing 100 μl PBS with gentamicin (100 μg ml⁻¹), and incubated at 37 °C for 1 h to kill extracellular bacteria. After incubation in gentamicin, the brains were washed three times in 500 μl PBS. At the final wash, the PBS also was plated onto nutrient agar plates to ensure any remaining bacteria had been killed. The washed brains were then resuspended in 100 μl fresh PBS containing SDS (0.5% final concentration) and vigorously vortexed to lyse the tissue. Aliquots of the lysate were plated on nutrient agar plates to determine the bacterial content.

**Preparation of isolated head capsules from locusts.** Locusts were ligated in the neck region using a loop of fine thread (Ameto spin polyester; Coats) placed between the head and thorax, and tied to allow the head capsule to be removed from the body by cutting just behind the ligature using fine scissors. Although ligation prevented leakage of material from the cut end of the gut, the back of the head capsule was sealed using a resin/wax mixture to ensure that no leakage of haemolymph from the head capsule could occur if the neck membrane had been damaged during ligation. In this way, the isolated head capsule contained an intact and undamaged brain, and the blood brain barrier remained intact within the capsule; damage to the CNS was limited to the cutting of the ventral nerve cord, the cut ends of which were sealed by the thread and the wax/resin mixture outside the isolated head capsule.

**Invasion of the locust brain by *E. coli*.** Isolated head capsules were allocated randomly into groups of ten, each head being
locust brain (Table 1). Thus all remaining assays were performed by injecting $5 \times 10^5$ bacteria per locust head capsule.

### E. coli K1 invaded locust brain in large numbers after an initial delay

Locust head capsules were injected with $5 \times 10^5$ E. coli K1, and brains dissected out at various intervals. E. coli K1 invaded the locust brain very rapidly, with low, but significant levels of bacteria being present in the brain even after only 15 min of incubation. Numbers of bacteria in the brain appeared to increase gradually (but this was not statistically significant) with time, until after about 2 h when there was an upsurge of invading bacteria and the numbers of bacteria invading the locust brain were significantly increased (comparing 15 min with 120 min $P=0.001$, with 150 min $P=0.0004$, with 180 min $P=0.006$, using paired t-test, one-tailed distribution) (Fig. 1). Beyond 2 h incubation, there appeared to be no significant further increase in numbers of bacteria in the brain (Fig. 1).

### The deletion of ompA abolished E. coli K1 invasion of locust brain

Previous studies in vivo using a newborn rat model have identified OmpA and Cnf1 as important bacterial determinants required for E. coli K1 meningitis. To test the requirement of these determinants for invasion of locust brain, assays were performed using isogenic gene deletion mutants of ompA and cnf1. The findings revealed that the ompA deletion mutant was significantly less able to invade locust brain compared with the parent strain ($P=1.04 \times 10^{-7}$ using paired t-test, two-tailed distribution) (Fig. 2). In fact, deletion of ompA reduced the levels of E. coli K1 invasion to levels similar to those found using the non-invasive K12 strain, HB101 (Fig. 2). Similarly, the cnf1 deletion mutant had a significantly reduced ability to invade locust brain compared with the parent strain ($P=0.0007$ using paired t-test, two-tailed distribution).

In addition to the aforementioned mutants, fimH and neuDB deletion mutants, as well as a rough LPS mutant derived from K1, were tested. As shown in Fig. 2, all mutants tested were significantly less invasive in locust brain compared with the parent strain ($P=0.0001$ for rough LPS mutant, $P=0.02$ for neuDB deletion mutant, $P=0.046$ for fimH deletion mutant, using paired t-test, two-tailed distribution).

Research over the last two decades has identified only a handful of determinants involved in E. coli K1 virulence. A likely explanation is the unavailability of functional assays that can screen large numbers of bacterial genes rapidly. With more than 500 kbp DNA specific to invasive E. coli, it is likely that many genes required for E. coli virulence remain unidentified. The present study was initiated to test whether E. coli meningitis can be modelled in locusts because E. coli K1 but not K12 kills locusts within 72 h of injection. Although it is not possible to be certain of the cause of death in K1-injected locusts, it is likely to be linked to invasion of the CNS by the bacteria. The results of this study add strong support to this argument because while K1 invades the locust brain very rapidly, K12 is not able to invade the brain even when present at a high concentration. It was surprising that when injected at high numbers ($10^9$) the recovery of K1 was reduced. A likely explanation may be the presence of extensive numbers of bacteria would result in damage that would allow gentamicin access to the inside of the brain, affecting intracerebral bacteria. Thus $5 \times 10^5$ was considered as an optimal number of bacteria for subsequent assays. In this study it is not

### Table 1. E. coli K1 invade locust brain in a concentration-dependent manner

Values are means $\pm$ SEM for the number of observations shown in parentheses.

<table>
<thead>
<tr>
<th>Initial inoculum of E. coli K1 (RS218) c.f.u.</th>
<th>No. of bacterial c.f.u. per brain (mean $\pm$ SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$10^4$ (6)</td>
<td>0</td>
</tr>
<tr>
<td>$5 \times 10^4$ (4)</td>
<td>$26.25 \pm 9^*$</td>
</tr>
<tr>
<td>$10^5$ (6)</td>
<td>$52.5 \pm 18^*$</td>
</tr>
<tr>
<td>$4 \times 10^5$ (2)</td>
<td>$490 \pm 21^*$</td>
</tr>
<tr>
<td>$5 \times 10^5$ (4)</td>
<td>$665 \pm 74^*$</td>
</tr>
<tr>
<td>$10^6$ (4)</td>
<td>$215 \pm 124$</td>
</tr>
</tbody>
</table>

*Indicates a significant difference ($P<0.05$ using paired t-test, one-tailed distribution) compared with the inoculum of $10^5$ bacterial c.f.u.
The finding that the rough LPS mutant exhibited significantly reduced invasion of locust brain appears in contrast to previous findings in the rat model that this mutant produces *E. coli* K1 meningitis at levels similar to the wild-type strain (Kim *et al.*, 1992). This may be due to difference between the models, i.e. locust *ex vivo* versus mammalian *in vivo*, but recent studies have determined that the rough LPS mutant used in the present study does not produce cytotoxicity of primary HBMEC *in vitro* (Kim *et al.*, 1992; unpublished findings). Why should LPS be critical in producing primary HBMEC cytotoxicity *in vitro*, and be of some importance in invading the locust brain *ex vivo*, yet trivial in invasion of rat CNS *in vivo*? Thus, it remains to be determined whether the locust *ex vivo* model described here is a good indicator of what might be found with mammalian models in this respect. Studies are in progress to address these questions.

Overall, the *ex vivo* model developed in this study is rapid, reproducible, relatively easy to perform, and can be used to study the process of *E. coli* K1 invasion of the CNS. While vertebrate model systems are seen as immediately more relevant, the locust model could be valuable in rapidly screening large numbers of genes/determinants of interest for *E. coli* K1 pathogenesis. In this way the locust model could generate potentially useful leads that can be tested subsequently in mammalian systems, thus reducing the numbers of mammals required overall in the study of bacterial infection.

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**REFERENCES**


