Using the Wax moth larva *Galleria mellonella* infection model to detect emerging waterborne bacterial pathogens

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Abstract

Climate change, changing farming practices, social and demographic changes and rising levels of antibiotic resistance are likely to lead to future increases in hard-to-treat bacterial infections resulting from contact with environmental water sources. Uncovering the prevalence and identity of waterborne pathogenic bacteria is key to assessing environmental transmission risks. We describe the first use of the Wax moth larva *Galleria mellonella*, a well-established model for the mammalian innate immune system, to selectively enrich and characterize pathogens from coastal environments in the South West of the U.K. Whole-genome sequencing of highly virulent isolates revealed amongst others a *Proteus mirabilis* strain carrying the *Salmonella* SGI1 genomic island not reported from the U.K. before and the recently described species *Vibrio injenensis* hitherto only reported from human patients in Korea. Our novel method has the power to detect novel bacterial pathogens in water as well as sediments that potentially pose a serious risk to public health.

1. Introduction

Microbial water safety is routinely assessed through the quantification of Faecal Indicator Bacteria (FIB) (WHO 2003). However, many FIB lineages are not associated with disease and there is no *a priori* reason to expect a relationship between FIB abundance and non-gastrointestinal disease (e.g. ear or skin infections). There are dozens of bacterial genera occurring in natural environments that are not primarily associated with human or animal faecal contamination but that are able to cause opportunistic infections (e.g. (Berg et al. 2005)). Alternatives to FIB such as quantification of pathogen-specific genes via molecular methods (Girones et al. 2010), flow cytometry (e.g. (Prest et al. 2013)) or isolation of specific pathogens (e.g. (Kaysner et al. 1987)) either are not linked to infection risk, are based on costly methodologies or are limited to a subset of ‘known knowns’. The current lack of a

direct screening method for the presence of pathogenic bacteria in water and sediment samples is therefore a major barrier to understanding drivers of virulence and ultimately infection risk.

We demonstrate the use of the Wax moth larva *Galleria mellonella* as a bioindicator for microbial water quality, and a means to selectively isolate and characterize pathogens. *G. mellonella* is a well-established model system for the mammalian innate immune system and has been used extensively to test for virulence in a range of human pathogens by quantifying survival rate after injection of a defined titre of a specific strain or mutant (Ramarao et al. 2012). Bacterial virulence in *Galleria* is positively correlated with virulence in mice (Brennan et al. 2002) as well as macrophages (Wand et al. 2011). Instead of quantifying the virulence of a specific bacterial clone, here we measure *Galleria* survival after injection with entire microbial communities from concentrated environmental water and sediment-wash samples. We isolate bacteria responsible for *Galleria* mortality and assess their pathogenic potential through whole-genome sequencing.

2. Material and Methods

2.1 Sample collection and processing

Eight sampling sites on the Fal estuary and English Channel coast near Falmouth (Cornwall, U.K.) were selected for water and sediment sampling. Four sites were estuarine and four truly coastal (Figure S1). Samples from each site were collected on 21 June 2017 and 6 July 2017. Each sampling effort consisted of collecting water (2 x 50 mL) and upper 1cm layer of sediment (~25 g) from all eight sites within a time span of two hours around low-tide. Samples were collected using sterile 50mL centrifuge tubes. All samples were kept on ice during transport and processed in laboratory within an hour from collection.

Each duplicate water sample was centrifuged at 500 rpm at 4°C for 15 minutes to pellet sediment particles. Supernatants were then transferred to sterile 50mL centrifuge tubes and spun down at 3500 rpm at 4°C for 30 minutes to pellet bacteria. Supernatant were then discarded and the pellet in each tube resuspended in 500µL of sterile M9 buffer. Resuspensions from duplicate samples were then combined in a 1.5 mL Eppendorf tube to obtain a total concentrated water sample of 1mL. Bacteria were extracted from sediments by adding 10g of sediment with 10mL of sterile M9 buffer. Samples were vortexed at 3000 rpm for 2 minutes, and then centrifuged at 500 rpm at 4°C for 15 minutes to pellet sediment particles. The supernatant was then centrifuged as above and resuspended in. 1mL of M9 buffer. All samples were kept at 4°C or on ice at all times.

2.2 Culturable Bacteria

100µL per water or sediment wash sample was plated onto both LB agar (Fisher BioReagents) and Coliform agar (Millipore Sigma) and incubated overnight at 37°C. CFU per plate were counted after 24 hr incubation. Colonies on coliform agar were scored as dark-blue ('*Escherichia coli*'), pink ('coliform'), or white/transparent ('other'). Units are reported in CFU/100mL for water samples and CFU/10g for sediment samples. Concentrated water and sediment wash samples were also analysed for bacterial load using flow cytometry (see Supplemental Methods).

2.3 *Galleria mellonella* assay for environmental samples

Final instar waxmoth (*Galleria mellonella*) larvae (~220mg each) were purchased from Livefood UK (http://www.livefood.co.uk), stored in the dark at 4°C, and used within two weeks (all experiments utilized larvae from the same batch). A 100µL Hamilton syringe (Sigma-Aldrich Ltd) with 0.3 x 13mm needle (BD Microlance 3) was used to inject larvae with 10µL of sample into the last left proleg. New sterile needles were used for injection of each sample, and the syringe was cleaned between sample injections with 70% ethanol and

sterile M9 buffer. All water and sediment washes (8 locations x 2 timepoints) were assayed in one experiment using 20 *Galleria* per sample. Three negative controls were used for each experiment: a no-injection control (to control for background larvae mortality), a buffer control in which larvae were injected with 10µL of M9 buffer (to control for impact of physical trauma), and a filter-sterilized sample control in which larvae were injected with filter-sterilized samples (0.22 µm syringe filters, Thermo Fisher) to account for any toxic chemicals present in the sample. Before injection, the *Galleria* larvae were separated into Petri dishes in groups of 20 and anesthetized by placing on ice for approximately 30 minutes before injection. After injection, Petri dishes were incubated at 37°C and inspected at 24, 48 and 72 hours post-injection to record morbidity and mortality. *Galleria* larvae were considered infected if they expressed dark pigmentation (melanisation) after inoculation. Larvae were scored as dead if they did not respond to touch stimuli by blunt sterile forceps.

2.4 *Galleria mellonella* assay for individual clones

To isolate and identify responsible pathogens, a subset of samples showing high *Galleria* mortality were inoculated and incubated again as described above. Eight live larvae showing signs of infection (melanisation) were selected at random from each sample group and dissected for haemocoel collection. Before haemocoel collection, the *Galleria* larvae were anesthetized on ice for 30 minutes and the site of dissection was sterilized with 70% ethanol. Sterile micro-scissors were used to remove the last left proleg, and a drop of haemocoel was allowed to exude from the larvae before being collected with a pipette. Approximately 5-15µL of haemocoel was collected per larva and diluted into 500µL of sterile M9 buffer in an Eppendorf tube. Serial dilutions of each sample were plated onto LB- and coliform agar plates and incubated overnight at 37°C. A single colony per sample was picked from these plates and grown for 24 hours in 5mL of LB broth at 37°C at 180 rpm. Overnight cultures were diluted in broth to a turbidity equivalent to a McFarland standard of

0.5 at 625 nm as measured by spectrophotometry (Bibby Scientific Limited, Staffordshire, UK). A serial dilution was plated on LB agar and incubated for 24 hours at 37°Cto obtain cell densities (CFU/mL). Individual clones were used to inoculate groups of 20 *Galleria* larvae with 10µL of 1x10^{2} CFU, 1x10^{4} CFU, and 1x10^{6} CFU. Larvae were incubated as described above and morbidity and mortality was recorded hourly after an initial 10-hour period for a total of 37 hours allowing for construction of survival plots and calculation of the 50% lethal dose value (LD_{50}).

2.5 Whole Genome Sequencing

DNA isolation, Illumina HiSeq sequencing and basic bioinformatics was performed through the MicrobeNG program in Birmingham, U.K. For details on all downstream bioinformatics analyses see Supplemental Methods.

3. Results and Discussion

Our survival assay shows that *Galleria* mortality after 72 hours varied widely between water and sediment samples collected at two dates from eight locations across Cornwall (U.K.), ranging from 5% to 95% (Fig. S2). Injection of buffer solution or filtered (0.22 µm) samples yielded zero mortality, demonstrating that injection was not harmful, and that samples did not contain lethal concentrations of pollutants or toxins. Mortality was largely congruent with FIB counts as well as total bacteria density (as quantified by flow cytometry and total viable counts on LB, Fig. S3), although there was substantial variation (Fig. S4 and Supplementary Results).

We chose four environmental samples exhibiting high (≥ 70%) *Galleria* mortality to isolate the clone(s) responsible for infection and reinoculated these samples to isolate bacteria from the haemocoel of infected, freshly killed larvae. All samples yielded a single colony type on each agar type, indicating that infections were (largely) clonal. A single clone was

picked for each sample, grown up and assayed using three inoculation densities ($1x10^2$ CFU, $1x10^4$ CFU, and $1x10^6$ CFU) (Fig. 1). All clones displayed high levels of virulence and were characterized using whole-genome sequencing (Fig. 1) (Supplemental Methods). We specifically focused on the identification of virulence- and antibiotic resistance genes (ARGs) as compiled in the VFDB (Chen et al. 2015) and CARD (Jia et al. 2016) databases respectively.

The first clone, isolated from estuarine mud (Supplemental Results) was identified as the enteric species *Proteus mirabilis*, most closely related to pathogenic strain HI4320 (Pearson et al. 2008) (Fig. 1A). Interestingly, this strain was found to carry a multidrug resistance genomic island (SGI1), first identified in an epidemic *Salmonella enterica* serovar Typhimurium clone in the 1990s (Boyd et al. 2001). This island has since been found in *P. mirabilis* isolated from human patients as well as from animals (Siebor and Neuwirth 2013) but to our knowledge not from *Proteus* strains isolated from natural environments. No virulence genes were found using a 90% similarity cut-off, but several were identified using a 75% cut-off (Table S2). The clone contains several antibiotic resistance genes (ARGs), including the tetracycline efflux protein *TetJ* and *AAC(6')-Ib7*, a plasmid-encoded aminoglycoside acetyltransferase (90% similarity cut off, Table S3).

The second clone, isolated from beach sand, was found to belong to *Vibrio injenensis*, a recently described species only known from two strains isolated from human patients in Korea (Paek et al. 2017) (Fig. 1B). The UK clone was 99% similar to the type strain M12-1144^T and carried 441 genes not present in the Korean strain. Both strains carry the *rtx* toxin operon (Table S4). Only two ARGs, including tetracycline resistance *tet34*, could be identified at a 75% similarity cut off in the UK isolate (Table S5). Unlike the Korean isolate, the UK strain appears to contain a toxin similar to the Zona occludens toxin (Zot) which is known to increase mammalian intestinal permeability (Fasano et al. 1995), with highest

similarity (74% amino acid similarity) to the fish pathogen *V. anguillarum* (Castillo et al. 2017). The isolation of this virulent clone is of particular interest as *Vibrio* species have been identified as high risk emerging infectious pathogens in Europe due to the effects of climate change (Lindgren et al. 2012).

The third clone *Pseudomonas aeruginosa* (Fig. 1C) isolated from seawater was found to belong to Sequence Type 667, which is represented by four genome-sequenced human pathogens. This clone carries an arsenal of virulence genes (228 at ≥90% nt identity; Table S6) including elastase (Gi et al. 2014) and Type II, III, IV and VI secretion systems. This *Pseudomonas aeruginosa* clone also carries a variety of ARGs (46 at ≥90% nt identity; Table S7), including triclosan- and multidrug efflux pumps and beta-lactamases, including *OXA50* conferring decreased susceptibility to ampicillin, ticarcillin, moxalactam and meropenem, and resistance to piperacillin-tazobactam and cephalotin (Girlich et al. 2004).

The fourth clone from estuarine mud was identified as *Escherichia coli* belonging to Phylogroup B2, specifically Sequence Type 3304 which is represented by three other isolates, from a human patient, a Mountain brushtail possum and one unknown (Fig. 1D). This isolate carries a range of virulence genes (Table S8), including *chuA*, *fyuA* and *vat* known to play a role in uropathogenicity (Müller et al. 2016), *set1A* associated with enteroaggregative *E. coli* (Mohamed et al. 2007) and *ibeA*, *OmpA* and *AslA* aiding brain microvascular epithelial cell invasion, known from avian pathogenic- and neonatal meningitis *E. coli* (Wang et al. 2011). This clone contains a range of ARGs, including multidrug- and aminoglycoside efflux pumps, a class C *ampC* beta-lactamase conferring resistance to cephalosporins and *pmrE* implicated in polymyxin resistance (Table S9).

4. Conclusions

Our study utilized the low-cost and ethically expedient *Galleria* infection model to directly measure the presence of pathogenic bacteria in environmental samples without any prior knowledge of identity. As expected, some samples with low FIB counts contained pathogenic bacteria and some samples with high FIB counts showed low *Galleria* mortality (Fig. S4). We note that of four pathogenic isolates, only one was a coliform and only two were gut-associated bacteria. Two out of the four isolates have not been reported from the U.K. before and potentially represent emerging infectious diseases. Emerging infectious diseases, of which a large proportion is caused by bacteria (estimated to be 54% (Jones et al. 2008) and 38% (Taylor et al. 2001)), pose a major threat to human health (Jones et al. 2008). Although most emerging bacterial pathogens have zoonotic origins, a large proportion of bacteria capable of infecting humans are free-living, for instance being associated with drinking water (Ford 1999) or recreational waters (Baker-Austin et al. 2013). Our results highlight the fact that transmission risks of waterborne bacterial pathogens extend beyond 'usual suspects' and includes environmental- and largely uncharacterized strains. Our relatively simple methods can provide a basis for future studies to detect pathogenic bacteria in aquatic- as well as other environments, to ultimately elucidate their ecological drivers and estimate human infection risk.

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Supplementary Information

Contains Supplementary Methods, Supplementary Results, nine Supplementary Tables and three Supplementary Figures.

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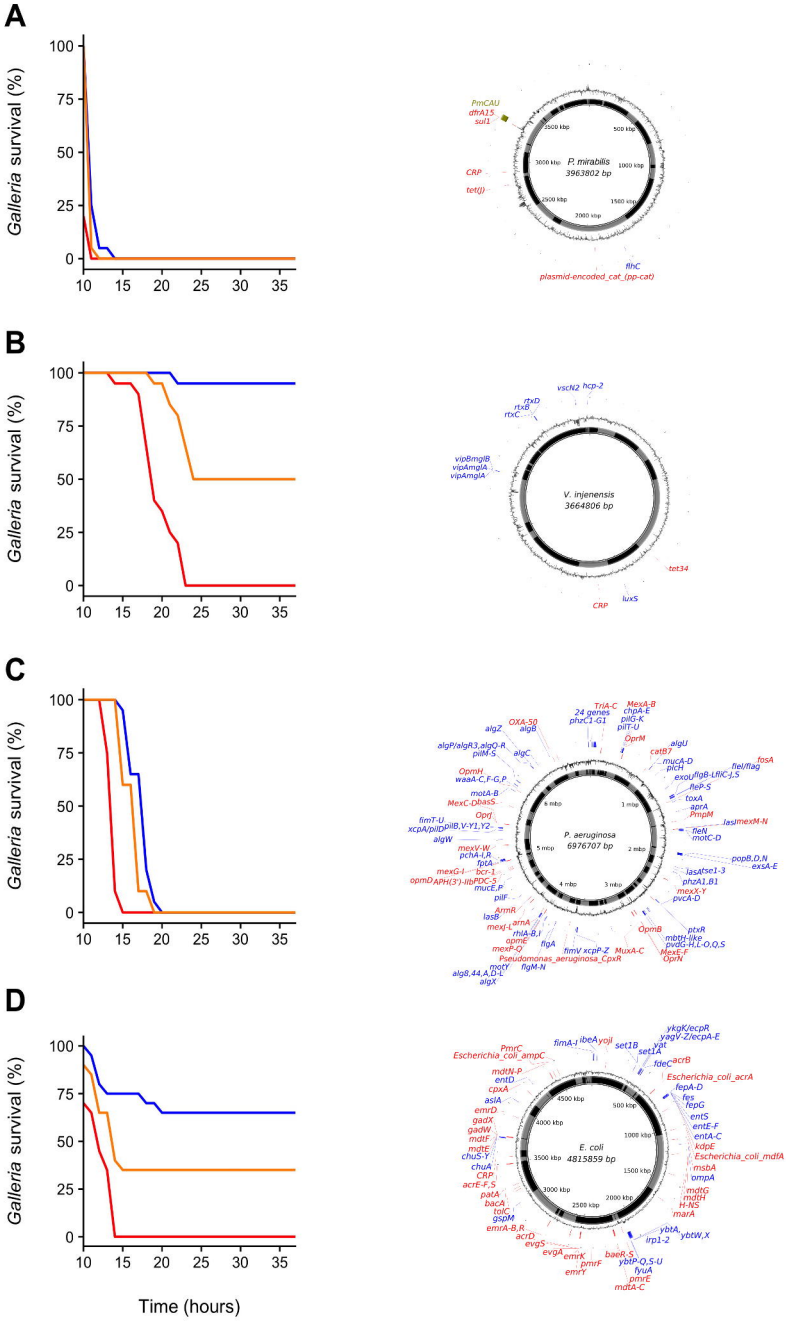
Figure Legend

Figure 1. Panels on the left show *Galleria mellonella* mortality after inoculation with individual bacterial clones originally isolated from *G. mellonella* larvae infected with environmental (whole-bacterial community) samples. Groups of 20 *Galleria* larvae were inoculated with 10µL of $1x10^2$ CFU (blue), $1x10^4$ CFU (orange) and $1x10^6$ CFU (red). Panels on the right show clone genome information (species name and genome size (middle), contigs (inner ring; grey and black), GC content (outer ring), virulence genes (blue) and ARGs (red) (≥75% nucleotide similarity used for *P. mirabilis* and *V. injenesis*; ≥90% similarity used for *P. aeruginosa* and *E. coli*; ≥80% coverage criterion for all four species). A: *Proteus mirabilis* (LD_{50}= $1x10^2$ CFU) (the genomic island SGI1-PmCAU is indicated in green), B: *Vibrio injenensis* (LD_{50}= $1x10^6$ CFU) (note that the absence of a closed draft genome means that contigs are randomly ordered), C: *Pseudomonas aeruginosa* (LD_{50}= $1x10^2$ CFU), D: *Escherichia coli* (LD_{50}= $1x10^4$ CFU).


A
Galleria survival (%)
100
75
50
25
0
10
15
20
25
30
35
PmCAU
dfrA15
sul1
CRP
tet(J)
P. mirabilis
3963802 bp
500 kbp
1000 kbp
1500 kbp
2000 kbp
2500 kbp
3000 kbp
3500 kbp
flhC
plasmid-encoded_cat_(pp-cat)
B
Galleria survival (%)
vscN2 hcp-2
rtxD
rtxB
rtxC
vipB/mglB
vipA/mglA
vipA/mglA
V. injenensis
3664806 bp
tet34
luxS
CRP
C
Galleria survival (%)
TriA-C
24 genes
phzC1-G1
MexA-B
chpA-E
pilG-K
pilT-U
OprM
algU
catB7
mucA-D
pilH
fosA
fleI/flaG
exoU/flgB-LflC-J,S
OXA-50
algB
algZ
algP/algR3,algQ-R
pilM-S
algC
OprnH
waaA-C,F-G,P
motA-B
MexC-DbasS
Opn
fimT-U
xcpA/pilD
pilB,V-Y1,Y2-
algW
mexY-W
pchA-I,R
fptA
mexG-I
bcr-1
opmD
APH(3')-IIb
ftdC-S
mucE,P
pilF
kimR
lasB
mexJ-I
arnA
rhlA-B,I
opmE
mexP-Q
flgA
Pseudomonas_aeruginosa_CpxR
motY
flgM-N
alg8,44,A,D-I
algX
P. aeruginosa
6976707 bp
1 mbp
2 mbp
3 mbp
4 mbp
5 mbp
6 mbp
fleP-S
toxA
aprA
PmpM
lasI/mexM-N
fleN
motC-D
popB,D,N
exsA-E
lasA/tse1-3
phzA1,B1
mexX-Y
pvcA-D
ptxR
mbtH-like
pvdG-H,L-O,Q,S
MexE-F
OprN
OpmB
MuxA-C
fimV xcpP-Z
D
Galleria survival (%)
PmrC
Escherichia_coli_ampC
mdtN-P
entD
cpxA
aslA
emrD
gadX
gadW
mdtF
mdtE
chuS-Y
chuA
CRP
acrE-F,S
patA
bacA
tolC
gspM
emrA-B,R
acrD
evgS
evgA
emrK
emrY
pmrF
baeR,S
ybtP-Q,S-U
fyuA
pmrE
mdtA-C
ibeA
yojI
fimA-I
set1B
set1A
yat
tdcC
acrB
ykgK/ecpR
yagV-Z/ecpA-E
Escherichia_coli_acrA
fepA-D
fes
fepG
entS
entE-F
entA-C
kdpE
Escherichia_coli_mdfA
msbA
ompA
mdtG
mdtH
H-NS
marA
ybtA,
ybtW,X
irp1-2
E. coli
4815859 bp
500 kbp
1000 kbp
1500 kbp
2000 kbp
2500 kbp
3000 kbp
3500 kbp
4000 kbp
4500 kbp
Time (hours)