Using the Wax moth larva *Galleria mellonella* infection model to detect emerging 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 opportunistic bacterial infections that are more difficult to treat. Uncovering the prevalence and identity of pathogenic bacteria in the environment 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 the environment that potentially pose a serious risk to public health.

Introduction

Emerging infectious diseases (EIDs) pose a major threat to human health (1). A large proportion of EIDs are caused by bacteria (estimated to be 54% (1) and 38% (2)). Although most emerging bacterial pathogens have zoonotic origins, a large proportion of infectious bacteria are free-living, for instance being associated with food (3), drinking water (4) or recreational waters (5). Microbial safety is routinely assessed through the quantification of Faecal Indicator Bacteria (FIB) (6). 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. (7)). Alternatives

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to FIB such as quantification of pathogen-specific genes via molecular methods (8), flow cytometry (e.g. (9)) or isolation of specific pathogens (e.g. (10)) 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 environmental 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 (11). Bacterial virulence in *Galleria* is positively correlated with virulence in mice (12) as well as macrophages (13). 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.

Material and Methods

<u>Sample collection and processing</u>

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^oC for 15 minutes to pellet sediment particles. Supernatants were then transferred to sterile 50mL centrifuge tubes and spun down at 3500 rpm at 4^oC 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^oC 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^oC or on ice at all times.

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^oC. 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).

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^oC, 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

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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 $37^{o}C$ 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.

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 $37^{o}C$. 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$_{50}$).

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.

Results

Our survival assay shows that *Galleria* mortality after 72 hours varied widely between both 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

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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 (14) and CARD (15) 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 (16) (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 (17). This island has since been found in *P. mirabilis* isolated from human patients as well as from animals (18) 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 (19) (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 (20), with highest similarity (74% amino acid

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similarity) to the fish pathogen *V. anguillarum* (21). 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 (22).

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 (23) 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 (24).

The fourth clone from estuarine mud was identified as *Escherichia coli* belonging to Phylogroup B2, specifically Sequence Type 3304, 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 (25), *set1A* associated with enteroaggregative *E. coli* (26) and *ibeA*, *OmpA* and *AslA* aiding brain microvascular epithelial cell invasion, known from avian pathogenic- and neonatal meningitis *E. coli* (27). 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).

Discussion

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

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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 EIDs. This highlights the fact that transmission risk extends 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 diverse environments, to ultimately elucidate their ecological drivers and estimate human infection risk.

Acknowledgments

Supplementary Information

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

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References

1.   Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL, et al. Global trends in emerging infectious diseases. *Nature*. 2008;451(7181):990.

2.   Taylor LH, Latham SM, Mark E. Risk factors for human disease emergence. *Philos Trans Royal Soc B: Biol Sci*. 2001;356(1411):983-9.

3.   Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H, et al. Food-borne diseases—the challenges of 20 years ago still persist while new ones continue to emerge. *Int J Food Microbiol*. 2010;139:S3-S15.

4.   Ford TE. Microbiological safety of drinking water: United States and global perspectives. *Env Health Persp*. 1999;107(Suppl 1):191.

5.   Baker-Austin C, Trinanes JA, Taylor NG, Hartnell R, Siitonen A, Martinez-Urtaza J. Emerging *Vibrio* risk at high latitudes in response to ocean warming. *Nature Clim Change*. 2013;3(1):73-7.

6.   World Health Organization. Guidelines for safe recreational water environments: Coastal and fresh waters (Vol 1). 2003.

7.   Berg G, Eberl L, Hartmann A. The rhizosphere as a reservoir for opportunistic human pathogenic bacteria. *Env Microbiol*. 2005;7(11):1673-85.

8.   Girones R, Ferrus MA, Alonso JL, Rodriguez-Manzano J, Calgua B, de Abreu Correˆa A, et al. Molecular detection of pathogens in water–the pros and cons of molecular techniques. *Water Res*. 2010;44(15):4325-39.

9.   Prest E, Hammes F, Kötzsch S, Van Loosdrecht M, Vrouwenvelder JS. Monitoring microbiological changes in drinking water systems using a fast and reproducible flow cytometric method. *Water Res*. 2013;47(19):7131-42.

10.   Kaysner C, Abeyta C, Wekell M, DePaola A, Stott R, Leitch J. Virulent strains of *Vibrio vulnificus* isolated from estuaries of the United States West Coast. *Applied Env Microbiol*. 1987;53(6):1349-51.

11.   Ramarao N, Nielsen-Leroux C, Lereclus D. The insect *Galleria mellonella* as a powerful infection model to investigate bacterial pathogenesis. *JoVE*. 2012(70).

12.   Brennan M, Thomas DY, Whiteway M, Kavanagh K. Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *FEMS Immunol Med Microbiol*. 2002;34(2):153-7.

13.   Wand ME, Müller CM, Titball RW, Michell SL. Macrophage and *Galleria mellonella* infection models reflect the virulence of naturally occurring isolates of *B. pseudomallei*, *B. thailandensis* and *B. oklahomensis*. *BMC Microbiol*. 2011;11(1):1.

14.   Chen L, Zheng D, Liu B, Yang J, Jin Q. VFDB 2016: hierarchical and refined dataset for big data analysis—10 years on. *Nucleic Acids Res*. 2015;44(D1):D694-D7.

15.   Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK, et al. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Res*. 2016:gkw1004.

16.   Pearson MM, Sebaihia M, Churcher C, Quail MA, Seshasayee AS, Luscombe NM, et al. Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and motility. *J Bac*. 2008;190(11):4027-37.

17.   Boyd D, Peters GA, Cloeckaert A, Boumedine KS, Chaslus-Dancla E, Imberechts H, et al. Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in phage type DT120 and serovar Agona. *J Bac*. 2001;183(19):5725-32.

18.   Siebor E, Neuwirth C. Emergence of *Salmonella* genomic island 1 (SGI1) among *Proteus mirabilis* clinical isolates in Dijon, France. *J Antimicrob Chemother*. 2013;68(8):1750-6.

19.   Paek J, Shin JH, Shin Y, Park I-S, Kim H, Kook J-K, et al. *Vibrio injenensis* sp. nov., isolated from human clinical specimens. *Antonie van Leeuwenhoek*. 2017;110(1):145-52.

20.   Fasano A, Fiorentini C, Donelli G, Uzzau S, Kaper JB, Margaretten K, et al. Zonula occludens toxin modulates tight junctions through protein kinase C-dependent actin reorganization, in vitro. *J Clin Investig*. 1995;96(2):710-20.

21.   Castillo D, Alvise PD, Xu R, Zhang F, Middelboe M, Gram L. Comparative Genome Analyses of *Vibrio anguillarum* Strains Reveal a Link with Pathogenicity Traits. *Msystems*. 2017;2(1):e00001-17.

22.   Lindgren E, Andersson Y, Suk JE, Sudre B, Semenza JC. Monitoring EU emerging infectious disease risk due to climate change. *Science*. 2012;336(6080):418-9.

23.   Gi M, Jeong J, Lee K, Lee K-M, Toyofuku M, Yong DE, et al. A drug-repositioning screening identifies pentetic acid as a potential therapeutic agent for suppressing the elastase-mediated virulence of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2014;58(12):7205-14.

24.   Girlich D, Naas T, Nordmann P. Biochemical characterization of the naturally occurring oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother*. 2004;48(6):2043-8.

25.   Müller A, Stephan R, Nüesch-Inderbinen M. Distribution of virulence factors in ESBL-producing *Escherichia coli* isolated from the environment, livestock, food and humans. *Science Total Envir*. 2016;541:667-72.

26.   Mohamed JA, Huang DB, Jiang Z-D, DuPont HL, Nataro JP, Belkind-Gerson J, et al. Association of putative enteroaggregative *Escherichia coli* virulence genes and biofilm

production in isolates from travelers to developing countries. *J Clin Microbiol.* 2007;45(1):121-6.
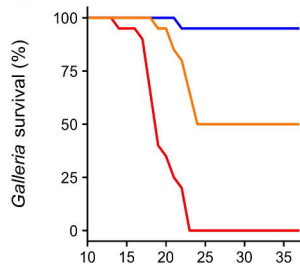
27.   Wang S, Niu C, Shi Z, Xia Y, Yaqoob M, Dai J, et al. Effects of *ibeA* deletion on virulence and biofilm formation of avian pathogenic *Escherichia coli*. *Infect Immun.* 2011;79(1):279-87.

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) ($\geq$75% nucleotide similarity used for *P. mirabilis* and *V. injenesis*; $\geq$90% similarity used for *P. aeruginosa* and *E. coli*; $\geq$80% coverage criterion for all four species). A: *Proteus mirabilis* ($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).

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A

Galleria survival (%)

Time (hours)

PmCAU
dfrA15
sul1

CRP
tet(J)

P. mirabilis
3963802 bp

flhC
plasmid-encoded_cat (pp-cat)

B

Galleria survival (%)

vscN2 *hcp-2*
rtxD
rtxB
rtxC

vipBmglB
vipAmglA
vipAmglA

V. injenensis
3664806 bp

tet34

CRP
luxS

C

Galleria survival (%)

TriA-C *MexA-B*
24 genes *chpA-E*
phzC1-G1 *pilG-K*
algZ *OXA-50* *pilT-U*
algB *OprM* *algU*
algP/algR3,algQ-R *catB7*
algC *mucA-D* *plcH*
OpmH *exoU* *flgB-LfllC-J,5* *fosA*
waaA-C,F-G,P *fleP-S* *flel/flag*
motB-A *toxA* *PmpM*
MexC-DbaeS *aprA* *las* *imexM-N*
fimT-U *fleN*
xcpA/pilD *pilB,V-Y1,Y2* *motC-D*
algW *popB,D,N*
mexV-W *lasAtse1-3* *exsA-E*
fptA *phzA1,B1*
OprT *mexX-Y*
mexG-I *bcr-1* *pvcA-D*
oprmD *APH(3')-IlbPDC-5* *ptxR*
mucE,P *ArnA* *mbtH-like*
pilF *mexI* *arnA* *pvdG-H,L-O,Q,S*
lasB *rhlA-B,I* *MexE-F*
mexP-Q *fimV* *xcpP-Z* *MuxA-C* *OprN*
Pseudomonas_aeruginosa_CpxR *OprB*
motY *flgM-N*
alg8,44,A,D-L
algX

P. aeruginosa
6976707 bp

6 mbo   1 mbo
5 mbo   2 mbo
4 mbo   3 mbo

D

Galleria survival (%)

fimA-I *ibeA* *yojl* *ykgK/ecpR*
Escherichia_coli_ampC *set1B* *yagV-Z/ecpA-E*
PmrC *set1A* *vat*
mdtN-P *fdeC* *acrB*
entD *Escherichia_coli_acrA*
cpxA *fepA-D* *acrA*
aslA *fes*
emrD *fepG*
gadX *entS*
gadW *entF-F*
mdtF *entA-C*
mdtE *kdpE*
chuS-Y *Escherichia_coli_mdfA*
chuA *msbA*
CRP *ompA*
acrE-F,S *mdtG*
patA *mdtH*
bacA *H-NS*
tolC *marA*
gspM
emrA-B,R
acrD *ybtA,*
evgS *lrp1-2* *ybtW,X*
evgA *baeR-S*
emrK *ybtP-Q,S-U*
pmrF *fyuA*
emrY *pmrE*
mdtA-C

E. coli
4815859 bp

4500 kbp   500 kbp
4000 kbp   1000 kbp
3500 kbp   1500 kbp
3000 kbp   2000 kbp
2500 kbp