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

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Abstract

Climate change, changing farming practices, rising levels of antibiotic resistance and social and demographic changes are likely to lead to future increases in community acquired opportunistic bacterial infections that are more difficult or impossible 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 characterise pathogens from environmental samples. Four highly virulent isolates isolated from coastal environments in the South West of the U.K. using this approach were whole-genome sequenced. A *Proteus mirabilis* strain was found to carry the *Salmonella* SGI1 genomic island, a combination which has emerged in the last ten years as a human and animal pathogen in hospitals and farms but has not been reported from the U.K. The recently described species *Vibrio injenensis* previously known only from human patients in Korea was isolated only for the third time. Pathogenic *Escherichia coli* and *Pseudomonas aeruginosa* strains were found to carry large numbers of virulence and antibiotic resistance genes. Our unbiased isolation method uncovered diverse virulent species, highlighting its power to detect potential emerging pathogens.

Results and Discussion

Emerging infectious diseases (EIDs) pose a major threat to human health (Jones et al 2008). A large proportion of EIDs are caused by bacteria (estimated to be 54% (Jones et al 2008) and 38% (Taylor et al 2001)). Although most emerging bacterial pathogens have zoonotic origins, a large proportion of infectious bacteria are free-living, for instance being associated with food (Newell et al 2010), drinking water (Ford 1999) or recreational waters (Baker-Austin et al 2013). Microbial 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

50 are not primarily associated with human or animal faecal contamination but that are able to cause
51 opportunistic infections (e.g. (Berg et al 2005)). Alternatives to FIB such as quantification of
52 pathogen-specific genes via molecular methods (Girones et al 2010), flow cytometry (e.g. (Prest
53 et al 2013)) or isolation of specific pathogens (e.g. (Kaysner et al 1987)) either are not linked to
54 infection risk, are based on costly methodologies or are limited to a subset of 'known knowns'.
55 The current lack of a direct screening method for the presence of pathogenic bacteria in
56 environmental samples is therefore a major barrier to understanding drivers of virulence and
57 ultimately infection risk.

58 We demonstrate the use of the Wax moth larva *Galleria mellonella* as a bioindicator for
59 microbial water quality, and a means to selectively isolate and characterise pathogens. *G.*
60 *mellonella* is a well-established model system for the mammalian innate immune system and has
61 been used extensively to test for virulence in a range of human pathogens by quantifying survival
62 rate after injection of a defined titre of a specific strain or mutant ((Ramarao et al 2012)). Bacterial
63 virulence in *Galleria* is positively correlated with virulence in mice (Brennan et al 2002) as well
64 as macrophages (Wand et al 2011). Instead of quantifying the virulence of a specific bacterial
65 clone, here we measure *Galleria* survival after injection with entire microbial communities from
66 concentrated environmental water and sediment-wash samples.

67 Water and sediment samples were collected on two sampling dates from eight coastal
68 locations around Falmouth (U.K.) (Fig. S1). 100 ml water samples were concentrated 100-fold by
69 centrifugation, sediment samples were vortexed in buffer (10g in 10 ml), after which they were
70 similarly concentrated by centrifugation (Supplemental Methods). 10 µl of sample thus processed
71 was injected into 20 *G. mellonella* larvae to record mortality after 24, 48 and 72 hours incubation
72 at 37°C. *Galleria* mortality after 72 hours varied widely between both water and sediment
73 samples, ranging from 5-95% (Fig. S2). Injection of buffer solution or filtered (0.22 µm) samples
74 yielded zero mortality, demonstrating that injection was not harmful, and that samples did not
75 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. Samples stored at 4°C were re-injected in *Galleria* and haemocoel of infected, freshly killed larvae was plated on both LB and coliform agar. 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 characterised 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 (Supplemental Methods).

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). 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, 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).

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. This highlights the fact that infection 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.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Information (SI)

The Supplementary Information contains Supplementary Methods, Supplementary Results, nine Supplementary Tables and three Supplementary Figures. WGS data will be submitted shortly.

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Figure 1

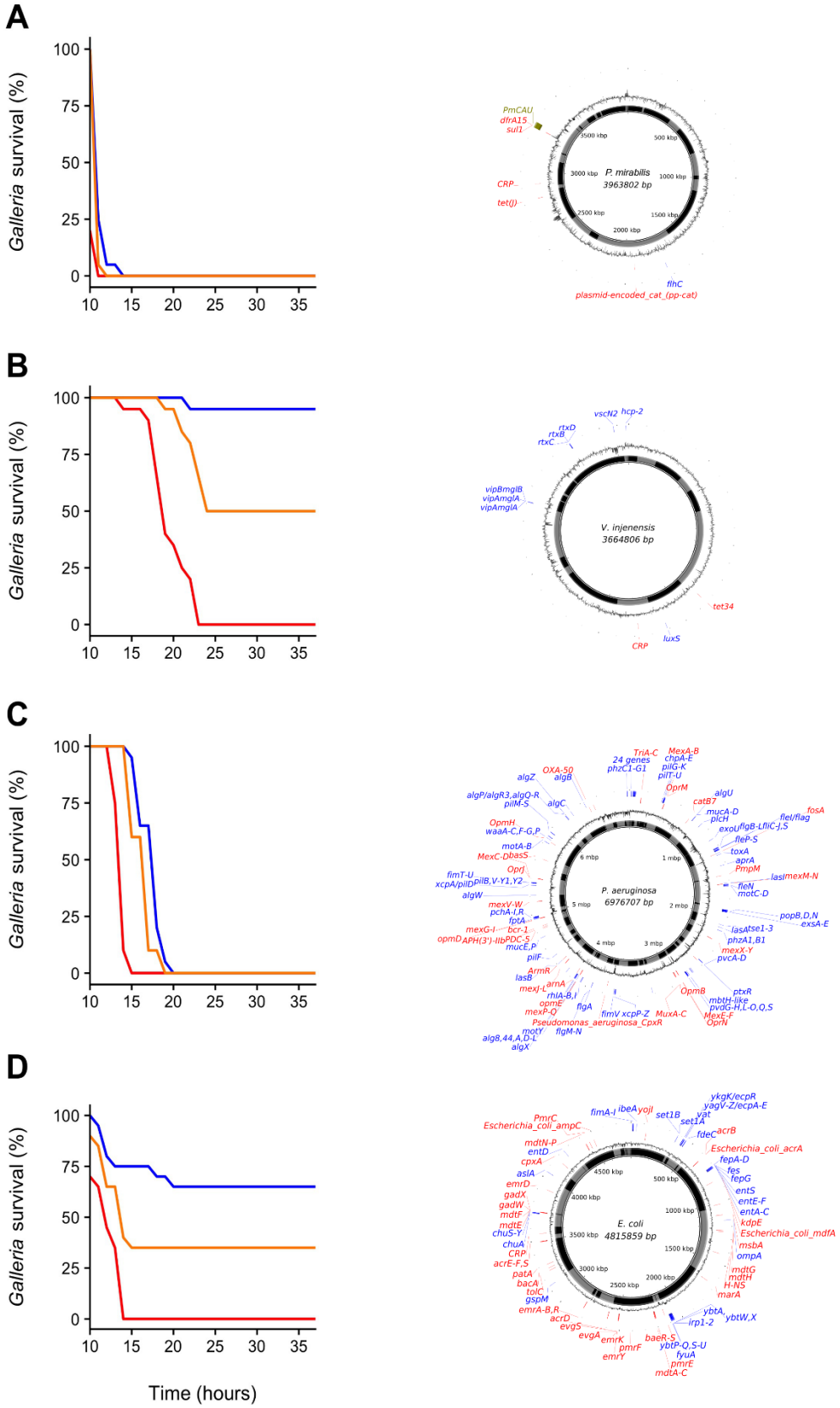

Figure 1. Panels on the left show *Galleria mellonella* mortality after inoculation with bacterial clones originally isolated from *G. mellonella* infected with environmental (whole-bacterial community) samples. Groups of 20 *Galleria* larvae were inoculated with 10µL of $1x10^2$ CFU (blue), $1x10^4$ 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).