bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

1 **Using the Wax moth larva *Galleria mellonella* infection model to detect emerging bacterial**
2 **pathogens**
3
4 Rafael J. Hernandez[1,2§], Elze Hesse[3§], Andrea Dowling[3], Nicola M. Coyle[4], Edward J. Feil[4], Will H.
5 Gaze[2] and Michiel Vos[2*]
6
7 1= Stony Brook School of Medicine, Department of Global Medical Education, Stony Brook
8 University, 101 Nicolls Road, Stony Brook, NY 11794-8434, U.S.A.
9 2= European Centre for Environment and Human Health, University of Exeter Medical School,
10 University of Exeter, Penryn Campus, TR10 9FE, U.K.
11 3= Department of Biosciences, University of Exeter, Penryn Campus, TR10 9FE, U.K.
12 4= The Milner Centre for Evolution, Department of Biology and Biochemistry, University of Bath,
13 Bath BA2 7AY, U.K.
14 §= joint first authors
15 *= corresponding author: m.vos@exeter.ac.uk
16
17
18
19 Key words: emerging infectious diseases, pathogens, virulence, antibiotic resistance,
20 *Galleria mellonella*, *Vibrio injenensis*, *Escherichia coli*, *Pseudomonas aeruginos*a, *Proteus mirabilis*
21
22

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

23 **Abstract**

24 Climate change, changing farming practices, rising levels of antibiotic resistance and social and
25 demographic changes are likely to lead to future increases in community acquired opportunistic
26 bacterial infections that are more difficult or impossible to treat. Uncovering the prevalence and
27 identity of pathogenic bacteria in the environment is key to assessing environmental
28 transmission risks. We describe the first use of the Wax moth larva *Galleria mellonella*, a well-
29 established model for the mammalian innate immune system, to selectively enrich and
30 characterise pathogens from environmental samples. Four highly virulent isolates isolated from
31 coastal environments in the South West of the U.K. using this approach were whole-genome
32 sequenced. A *Proteus mirabilis* strain was found to carry the *Salmonella* SGI1 genomic island, a
33 combination which has emerged in the last ten years as a human and animal pathogen in hospitals
34 and farms but has not been reported from the U.K. The recently described species *Vibrio injenensis*
35 previously known only from human patients in Korea was isolated only for the third time.
36 Pathogenic *Escherichia coli* and *Pseudomonas aeruginosa* strains were found to carry large
37 numbers of virulence and antibiotic resistance genes. Our unbiased isolation method uncovered
38 diverse virulent species, highlighting its power to detect potential emerging pathogens.

39

40 **Results and Discussion**

41 Emerging infectious diseases (EIDs) pose a major threat to human health (Jones et al
42 2008). A large proportion of EIDs are caused by bacteria (estimated to be 54% (Jones et al 2008)
43 and 38% (Taylor et al 2001)). Although most emerging bacterial pathogens have zoonotic origins,
44 a large proportion of infectious bacteria are free-living, for instance being associated with food
45 (Newell et al 2010), drinking water (Ford 1999) or recreational waters (Baker-Austin et al 2013).
46 Microbial safety is routinely assessed through the quantification of Faecal Indicator Bacteria (FIB)
47 (WHO 2003). However, many FIB lineages are not associated with disease and there is no *a priori*
48 reason to expect a relationship between FIB abundance and non-gastrointestinal disease (e.g. ear
49 or skin infections). There are dozens of bacterial genera occurring in natural environments that

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

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

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

76 counts as well as total bacteria density (as quantified by flow cytometry and total viable counts
77 on LB, Fig. S3), although there was substantial variation (Fig. S4 and Supplementary Results).
78 We chose four environmental samples exhibiting high (≥ 70%) *Galleria* mortality to
79 isolate the clone(s) responsible for infection. Samples stored at 4°C were re-injected in *Galleria*
80 and haemocoel of infected, freshly killed larvae was plated on both LB and coliform agar. All
81 samples yielded a single colony type on each agar type, indicating that infections were (largely)
82 clonal. A single clone was picked for each sample, grown up and assayed using three inoculation
83 densities ($1x10^2$ CFU, $1x10^4$ CFU, and $1x10^6$ CFU) (Fig. 1). All clones displayed high levels of
84 virulence and were characterised using whole-genome sequencing (Fig. 1) (Supplemental
85 Methods). We specifically focused on the identification of virulence- and antibiotic resistance
86 genes (ARGs) as compiled in the VFDB (Chen et al 2015) and CARD (Jia et al 2016) databases
87 respectively (Supplemental Methods).
88 The first clone, isolated from estuarine mud (Supplemental Results) was identified as the
89 enteric species *Proteus mirabilis*, most closely related to pathogenic strain HI4320 (Pearson et al
90 2008) (Fig. 1A). Interestingly, this strain was found to carry a multidrug resistance genomic island
91 (SGI1), first identified in an epidemic *Salmonella enterica* serovar Typhimurium clone in the
92 1990s (Boyd et al 2001). This island has since been found in *P. mirabilis* isolated from human
93 patients as well as from animals (Siebor and Neuwirth 2013) but to our knowledge not from
94 *Proteus* strains isolated from natural environments. No virulence genes were found using a 90%
95 similarity cut-off, but several were identified using a 75% cut-off (Table S2). The clone contains
96 several antibiotic resistance genes (ARGs), including the tetracycline efflux protein *TetJ* and
97 *AAC(6')-Ib7*, a plasmid-encoded aminoglycoside acetyltransferase (90% similarity cut off, Table
98 S3).
99 The second clone, isolated from beach sand, was found to belong to *Vibrio injenensis*, a
100 recently described species only known from two strains isolated from human patients in Korea
101 (Paek et al 2017) (Fig. 1B). The UK clone was 99% similar to the type strain M12-1144^T and
102 carried 441 genes not present in the Korean strain. Both strains carry the *rtx* toxin operon (Table

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

103 S4). Only two ARGs, including tetracycline resistance *tet34*, could be identified at a 75% similarity
104 cut off in the UK isolate (Table S5). The isolation of this virulent clone is of particular interest as
105 *Vibrio* species have been identified as high risk emerging infectious pathogens in Europe due to
106 the effects of climate change (Lindgren et al 2012).

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

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

125 Our study utilized the low-cost and ethically expedient *Galleria* infection model to directly
126 measure the presence of pathogenic bacteria in environmental samples without any prior
127 knowledge of identity. As expected, some samples with low FIB counts contained pathogenic
128 bacteria and some samples with high FIB counts showed low *Galleria* mortality (Fig. S4). We note
129 that of four pathogenic isolates, only one was a coliform and only two were gut-associated

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

130 bacteria. Two out of the four isolates have not been reported from the U.K. before and potentially
131 represent emerging infectious diseases. This highlights the fact that infection risk extends beyond
132 'usual suspects' and includes environmental- and largely uncharacterized strains. Our relatively
133 simple methods can provide a basis for future studies to detect pathogenic bacteria in diverse
134 environments, to ultimately elucidate their ecological drivers and estimate human infection risk.
135

136 **Acknowledgments**
137 This work was supported by a Stony Brook Medicine International Research Fellowship awarded
138 to RJH. We thank Francisca Garcia-Garcia for help with flow cytometry.
139

140 **Conflict of Interest**
141 The authors declare no conflict of interest.
142

143 **Supplementary Information (SI)**
144 The Supplementary Information contains Supplementary Methods, Supplementary Results, nine
145 Supplementary Tables and three Supplementary Figures. WGS data will be submitted shortly.

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

146 **References**

147

148 Baker-Austin C, Trinanes JA, Taylor NG, Hartnell R, Siitonen A, Martinez-Urtaza J (2013).
149 Emerging *Vibrio* risk at high latitudes in response to ocean warming. *Nat Clim Change* **3:** 73-77.

150

151 Berg G, Eberl L, Hartmann A (2005). The rhizosphere as a reservoir for opportunistic human
152 pathogenic bacteria. *Env Microbiol* **7:** 1673-1685.

153

154 Boyd D, Peters GA, Cloeckaert A, Boumedine KS, Chaslus-Dancla E, Imberechts H *et al* (2001).
155 Complete nucleotide sequence of a 43-kilobase genomic island associated with the multidrug
156 resistance region of *Salmonella enterica* serovar Typhimurium DT104 and its identification in
157 phage type DT120 and serovar Agona. *J Bac* **183:** 5725-5732.

158

159 Brennan M, Thomas DY, Whiteway M, Kavanagh K (2002). Correlation between virulence of
160 *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *FEMS Immunol Med Microbiol*
161 **34:** 153-157.

162

163 Chen L, Zheng D, Liu B, Yang J, Jin Q (2015). VFDB 2016: hierarchical and refined dataset for big
164 data analysis—10 years on. *Nucleic Acids Res* **44:** D694-D697.

165

166 Ford TE (1999). Microbiological safety of drinking water: United States and global perspectives.
167 *Env Health Perspect* **107:** 191.

168

169 Gi M, Jeong J, Lee K, Lee K-M, Toyofuku M, Yong DE *et al* (2014). A drug-repositioning screening
170 identifies pentetic acid as a potential therapeutic agent for suppressing the elastase-mediated
171 virulence of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **58:** 7205-7214.

172

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

173 Girlich D, Naas T, Nordmann P (2004). Biochemical characterization of the naturally occurring
174 oxacillinase OXA-50 of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* **48:** 2043-2048.
175
176 Girones R, Ferrus MA, Alonso JL, Rodriguez-Manzano J, Calgua B, de Abreu Correˆa A *et al* (2010).
177 Molecular detection of pathogens in water–the pros and cons of molecular techniques. *Water Res*
178 **44:** 4325-4339.
179
180 Jia B, Raphenya AR, Alcock B, Waglechner N, Guo P, Tsang KK *et al* (2016). CARD 2017: expansion
181 and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids*
182 *Res***:** gkw1004.
183
184 Jones KE, Patel NG, Levy MA, Storeygard A, Balk D, Gittleman JL *et al* (2008). Global trends in
185 emerging infectious diseases. *Nature* **451:** 990.
186
187 Kaysner C, Abeyta C, Wekell M, DePaola A, Stott R, Leitch J (1987). Virulent strains of *Vibrio*
188 *vulnificus* isolated from estuaries of the United States West Coast. *Appl Env Microbiol* **53:** 1349-
189 1351.
190
191 Lindgren E, Andersson Y, Suk JE, Sudre B, Semenza JC (2012). Monitoring EU emerging infectious
192 disease risk due to climate change. *Science* **336:** 418-419.
193
194 Mohamed JA, Huang DB, Jiang Z-D, DuPont HL, Nataro JP, Belkind-Gerson J *et al* (2007).
195 Association of putative enteroaggregative *Escherichia coli* virulence genes and biofilm production
196 in isolates from travelers to developing countries. *J Clin Microbiol* **45:** 121-126.
197

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

198 Müller A, Stephan R, Nüesch-Inderbinen M (2016). Distribution of virulence factors in ESBL-
199 producing *Escherichia coli* isolated from the environment, livestock, food and humans. *Sci Total*
200 *Env* **541:** 667-672.
201
202 Newell DG, Koopmans M, Verhoef L, Duizer E, Aidara-Kane A, Sprong H *et al* (2010). Food-borne
203 diseases—the challenges of 20 years ago still persist while new ones continue to emerge. *Int J*
204 *Food Microbiol* **139:** S3-S15.
205
206 World Health Organization (2003). Guidelines for safe recreational water environments: Coastal
207 and fresh waters. Vol. 1. World Health Organization.
208
209 Paek J, Shin JH, Shin Y, Park I-S, Kim H, Kook J-K *et al* (2017). *Vibrio injenensis* sp. nov., isolated
210 from human clinical specimens. *Antonie van Leeuwenhoek* **110:** 145-152.
211
212 Pearson MM, Sebaihia M, Churcher C, Quail MA, Seshasayee AS, Luscombe NM *et al* (2008).
213 Complete genome sequence of uropathogenic *Proteus mirabilis*, a master of both adherence and
214 motility. *J Bac* **190:** 4027-4037.
215
216 Prest E, Hammes F, Kötzsch S, Van Loosdrecht M, Vrouwenvelder JS (2013). Monitoring
217 microbiological changes in drinking water systems using a fast and reproducible flow cytometric
218 method. *Water Res* **47:** 7131-7142.
219
220 Ramarao N, Nielsen-Leroux C, Lereclus D (2012). The insect *Galleria mellonella* as a powerful
221 infection model to investigate bacterial pathogenesis. *JoVE* (70).
222
223 Siebor E, Neuwirth C (2013). Emergence of Salmonella genomic island 1 (SGI1) among *Proteus*
224 *mirabilis* clinical isolates in Dijon, France. *J Antimicrob Chemother* **68:** 1750-1756.

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

225

226 Taylor LH, Latham SM, Mark E (2001). Risk factors for human disease emergence. *Philos Trans R*
227 *Soc B: Biol Sci* **356:** 983-989.
228
229 Wand ME, Müller CM, Titball RW, Michell SL (2011). Macrophage and *Galleria mellonella* infection
230 models reflect the virulence of naturally occurring isolates of *B. pseudomallei*, *B. thailandensis* and
231 *B. oklahomensis*. *BMC Microbiol* **11:** 1.
232
233 Wang S, Niu C, Shi Z, Xia Y, Yaqoob M, Dai J *et al* (2011). Effects of *ibeA* deletion on virulence and
234 biofilm formation of avian pathogenic *Escherichia coli*. *Infect Immun* **79:** 279-287.
235

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

236 **Figure 1**

A

B

C

D

Time (hours)

237

bioRxiv preprint doi: https://doi.org/10.1101/327015; this version posted May 21, 2018. The copyright holder for this preprint (which was not certified by peer review) is the author/funder. All rights reserved. No reuse allowed without permission.

238 Figure 1. Panels on the left show *Galleria mellonella* mortality after inoculation with bacterial
239 clones originally isolated from *G. mellonella* infected with environmental (whole-bacterial
240 community) samples. Groups of 20 *Galleria* larvae were inoculated with 10μL of $1x10^2$ CFU (blue),
241 $1x10^4$ CFU (orange) and $1x10^6$ CFU (red). Panels on the right show clone genome information
242 (species name and genome size (middle), contigs (inner ring; grey and black), GC content (outer
243 ring), virulence genes (blue) and ARGs (red) (≥75% nucleotide similarity used for *P. mirabilis* and
244 *V. injenesis*; ≥90% similarity used for *P. aeruginosa* and *E. coli*; ≥80% coverage criterion for all
245 four species). A: *Proteus mirabilis* (LD_{50}= $1x10^2$ CFU) (the genomic island SGI1-PmCAU is
246 indicated in green), B: *Vibrio injenensis* (LD_{50}= $1x10^6$ CFU) (note that the absence of a closed draft
247 genome means that contigs are randomly ordered), C: *Pseudomonas aeruginosa* (LD_{50}= $1x10^2$
248 CFU), D: *Escherichia coli* (LD_{50}= $1x10^4$ CFU).