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REVISED

Exposure to norepinephrine enhances *Brachyspira pilosicoli* growth, attraction to mucin and attachment to Caco-2 cells

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Running title: Norepinephrine and *Brachyspira pilosicoli*

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22 **Summary**

23 *Brachyspira pilosicoli* is an anaerobic intestinal spirochaete that colonises the large
24 intestine of a variety of species of birds and mammals, including human beings.
25 Colonisation may result in a mild colitis and diarrhoea in a condition known as
26 “intestinal spirochaetosis”. The catecholamine norepinephrine (NE), which is known to
27 influence the behaviour of many bacterial species, may be present in the colon. The
28 purpose of the current study was to determine whether exposure of *B. pilosicoli* to NE
29 would influence its *in vitro* behaviour in assays that may reflect *in vivo* colonisation
30 potential. *B. pilosicoli* strain 95/1000 was used in all the assays. Addition of NE at a
31 concentration of 0.05 mM to *B. pilosicoli* growing in anaerobic broth significantly
32 increased spirochaete numbers after four days incubation. The effect of higher
33 concentrations of NE was not significant. Exposure to 0.05 mM NE, but not to higher
34 concentrations, also resulted in significantly more spirochaete cells entering capillary
35 tubes containing 4% porcine gastric mucin than occurred with untreated cultures.
36 When NE was added to chemotaxis buffer in capillary tubes significantly more
37 spirochaetes were attracted to the buffer containing NE concentrations of 0.1, 0.5 and
38 1.0 mM than to 0.05 mM, or when no NE was added. Exposure of *B. pilosicoli* cultures
39 to 0.05 mM NE prior to incubation with Caco-2 monolayers resulted in more
40 attachment to the monolayer than occurred with non-exposed cultures. These results
41 show that at higher concentrations NE acts as a chemoattractant for *B. pilosicoli*, and at
42 0.05 mM it increases the spirochaete’s growth rate, attraction to mucin, and its rate of
43 attachment to cultured enterocytes. These activities are likely to enhance the ability of
44 *B. pilosicoli* to colonise, and may be induced by conditions that increase NE
45 concentrations in the intestinal tract, such as the stresses associated with crowding.

46

47 **INTRODUCTION**

48 *Brachyspira pilosicoli* is an anaerobic intestinal spirochaete that colonises the large intestines
49 of many species of birds and mammals, including human beings. A frequent feature of the
50 colonization is the end-on attachment of spirochaete cells to the luminal surface of colonic
51 and rectal epithelial cells, in a condition called “intestinal spirochaetosis” (IS). Infections
52 with *B. pilosicoli* are common amongst intensively farmed pigs and chickens, where they
53 cause diarrhoea and reduced production (Hampson & Duhamel, 2006; Hampson & Swayne,
54 2008). Colonisation also is common in people living in crowded and unhygienic conditions in
55 developing countries (Trott *et al.*, 1997; Margawani *et al.*, 2004; Nelson *et al.*, 2009), as well
56 as in homosexual males and HIV positive individuals in developed countries (Law *et al.*,
57 1994; Trivett-Moore *et al.*, 1998). In some studies colonisation in humans has been found to
58 be significantly associated with chronic diarrhoea, failure to thrive and being underweight
59 (Brooke *et al.*, 2006).

60

61 Catecholamines, including norepinephrine (NE), are known to have important effects on the
62 growth and behaviour of a range of pathogenic bacterial species (eg Bansal *et al.*, 2007;
63 Cogan *et al.*, 2007; Doherty *et al.*, 2009). NE is present in the intestinal lumen, where it
64 arrives driven by diffusion down a concentration gradient from the blood (Lyte & Bailey
65 1997). Consequently, *B. pilosicoli* is likely to be exposed to NE in the colon. The aim of the
66 current study was to investigate whether NE exposure can influence *B. pilosicoli* in its *in*
67 *vitro* growth rate, attraction to mucin and attachment to Caco-2 cell monolayers. These *in*
68 *vitro* activities were chosen for study as they are likely to reflect the capacity of the
69 spirochaete to colonise *in vivo*. Strain 95/1000 was used because its genome has been
70 sequenced (Wanchanthuck *et al.*, 2010) and it has been used in a number of published

71 studies, including studies of motility and chemotaxis (Naresh & Hampson 2010), and
72 attachment to Caco-2 cells (Naresh *et al.*, 2009).

73

74 **METHODS**

75 **Preparation of norepinephrine stock solution.** Stock solutions (0.01 M) of norepinephrine
76 bitartrate salt (NE) (Sigma-Aldrich, St. Louis, USA) were prepared in phosphate buffered
77 saline (PBS) and were sterilised by filtration. The stock solutions were prepared just before
78 the start of each experiment, and were held in a dark glass vessel to avoid exposure to light.

79

80 **Spirochaete strain and cultivation.** *Brachyspira pilosicoli* strain 95/1000, which was
81 originally isolated from a pig with porcine intestinal spirochaetosis in a Western Australian
82 herd, was obtained as frozen stock from the culture collection held at the Australian
83 Reference Centre for Intestine Spirochaetes, School of Veterinary and Biomedical Sciences,
84 Murdoch University. The cells were thawed, and grown at 39°C in Kunkle's pre-reduced
85 anaerobic broth containing 2% (v/v) foetal bovine serum and 1% (v/v) ethanolic cholesterol
86 solution (Kunkle *et al.*, 1986). The growth of the spirochaete and absence of contamination
87 were monitored by examining aliquots under a phase contrast microscope. The cultures were
88 harvested in early log-phase, when the spirochaetes were actively motile, and were
89 enumerated by direct counting in a counting chamber under a phase contrast microscope. For
90 counting, duplicate preparations were used, and spirochaetes were counted in 48 squares by
91 one operator.

92

93 **Effect of NE on the growth of *B. pilosicoli* 95/1000.** A set of 20 mL glass tubes each
94 containing nine mL of Kunkle's anaerobic broth medium were prepared, wrapped with

95 aluminium foil to keep them dark, and each was seeded with 0.5 mL of a broth culture of *B.*
96 *pilosicoli* 95/1000 at a concentration of 10^7 cells per mL. A fresh stock solution of NE was
97 prepared and added to the tubes to achieve concentrations of 0.05, 0.1, 0.5 and 1 mM NE. An
98 equivalent volume of sterile PBS was added to the control tubes. Six replicates of each NE
99 concentration and the NE-free control broths were used in each test. The tubes were
100 incubated on a rocking platform at 39°C for four days, and then aliquots were removed and
101 the spirochaetes were counted. Six biological replicates were used.

102

103 **Chemotaxis assays.** Chemotaxis assays were undertaken using glass haematocrit capillary
104 tubes filled either with chemotaxis buffer (0.01 M potassium phosphate buffer [pH 7.0], 0.2
105 mM L-cysteine hydrochloride) or 4% porcine gastric mucin type II (Sigma Aldrich) prepared
106 in chemotaxis buffer, as previously described (Naresh & Hampson, 2010).

107

108 **NE added to the broth culture.** NE was added to active cultures of *B. pilosicoli* 95/1000
109 (10^8 /mL) to obtain final concentrations of 0.05, 0.1, 0.5 and 1 mM. For each concentration
110 six capillary tubes were allocated; these were filled with 4% mucin, the top ends were sealed
111 with plasticine and they were hung vertically with their lower ends submerged in the
112 appropriate *B. pilosicoli* 95/1000 culture in 48 well round bottomed tissue culture plates.
113 These were incubated at 39°C for 90 minutes in a CO₂ incubator. The outside of each tube
114 was then wiped dry with a sterile tissue and it was placed upright into a 200 µl eppendorf
115 tube. The top of the capillary tube was gently broken and the contents were collected. The
116 solution was serially diluted in PBS and the spirochaetes were counted. Six biological
117 replicates were used.

118

119 **NE added to chemotaxis buffer.** Fresh NE was added to chemotaxis buffer to final
120 concentrations of 0.05, 0.1, 0.5 and 1 mM. Six capillary tubes were filled with buffer for each
121 of the NE concentrations. The chemotaxis assay was conducted as described above, again
122 with six biological replicates.

123

124 **Effect of NE on *B. pilosicoli* attachment to Caco-2 cells.** The attachment assays were
125 conducted as previously described (Naresh *et al.*, 2009), with three biological replicates.
126 Briefly, two-week-old confluent Caco-2 cell monolayers were grown on 10 mm round glass
127 coverslips in 48 well plates at 37°C. A fresh mid-log phase broth culture of *B. pilosicoli* strain
128 95/1000 (10^8 cells/mL) was harvested and NE was added to an aliquot of the culture to give a
129 final dilution of 0.05 mM. One mL volumes of this culture or the culture without NE were
130 pipetted into the wells and incubated for 2, 4 and 6 h. Three wells were allocated for each
131 time interval. The wells then were washed three times with PBS to remove unattached
132 spirochaetes and the coverslips were removed and processed for scanning electron
133 microscopy (SEM), as previously described (Naresh *et al.*, 2009).

134

135 **Analysis of data.** *B. pilosicoli* growth in broth containing different concentrations of NE was
136 compared by one way analysis of variance (ANOVA) using SPSS for Windows. ANOVA
137 also was used to compare the numbers of spirochaete cells recovered from the capillary tubes
138 in the chemotaxis assays. The degree of *B. pilosicoli* attachment to Caco-2 cells as observed
139 under the SEM was recorded subjectively.

140

141 **RESULTS AND DISCUSSION**

142 **Effect of NE on growth of *B. pilosicoli***

143 The addition of NE to the *B. pilosicoli* culture resulted in a significant ($P > 0.002$) increase in
144 growth only with 0.05 mM NE (Fig. 1). With this concentration the number of spirochaetes
145 was just over 8×10^7 per mL compared to approximately 5×10^7 per mL for the non-exposed
146 culture. The number of bacteria in the latter cultures had increased approximately ten-fold
147 during the four-day incubation. The number of bacteria also was higher with the 0.1 mM NE
148 concentration than with the non-exposed control culture, but the difference was not
149 statistically significant. The number of bacteria in the two remaining NE concentrations did
150 not differ significantly from the control.

151 **[Fig. 1 about here]**

152 The increase in cell numbers that occurred following exposure to 0.05 mM NE was not large,
153 and other bacterial species have shown far greater increases in growth after NE exposure. For
154 example, *Campylobacter jejuni* showed a 50-fold increase in growth following NE exposure
155 (Cogan *et al.*, 2007). Nevertheless, *B. pilosicoli* is a slow-growing anaerobe and any increase
156 in growth rate could enhance its capacity to colonise the large intestine.

157

158 **Effect of NE on the attraction of *B. pilosicoli* to mucin**

159 The results of the assays where *B. pilosicoli* was exposed to different concentrations of NE at
160 the time they were added to the mucin attraction assay are summarized in Fig. 2. The culture
161 exposed to the lowest NE concentration (0.05 mM) showed significantly ($P > 0.02$) greater
162 attraction to 4% mucin than the control that was not exposed to NE. No other differences
163 were statistically significant.

164 **[Fig. 2 about here]**

165 Previously it has been shown that the attraction of *B. pilosicoli* 95/1000 to 4% mucin is likely
166 to involve elements of both chemotaxis and viscotaxis (Naresh & Hampson). The rapid

167 change in the spirochaete's responsiveness to the mucin following exposure to 0.05 mM NE
168 may involve either increased sensitivity to chemotactic signals and/or an increased motility
169 and motion efficiency that allowed it to enter the mucin solution more rapidly.

170

171 **Attraction of *B. pilosicoli* to NE in chemotaxis buffer**

172 The effect of addition of NE to the chemotaxis buffer on the number of *B. pilosicoli* cells
173 entering the buffer is shown in Fig. 3. The 0.1, 0.5 and 1 mM NE concentrations attracted
174 significantly more spirochaetes than the control chemotaxis buffer ($P > 0.004$). Spirochaete
175 numbers did not differ significantly at these three NE concentrations. The number of bacteria
176 at the lowest NE level (0.05 mM) was significantly less ($P > 0.03$) than in the other three NE
177 concentrations, and did not differ significantly from the control without NE. The most likely
178 explanation for these results was that the NE acted as a chemoattractant for the spirochaete,
179 with this activity being saturated at 0.01 mM NE. Similar chemoattractant responses to NE
180 occur with other bacterial species (Bansal *et al.*, 2007; Bearson & Bearson 2008).
181 Interestingly, there was approximately one log fewer spirochaetes in the capillary tubes
182 containing NE than in those tubes containing mucin where the spirochaetes had not been
183 exposed to NE. Hence the mucin appeared to be a stronger attractant than the NE.

184

[Fig. 3 about here]

185 **Attachment assays with a culture of *B. pilosicoli* exposed to NE**

186 The *B. pilosicoli* cultures that either had or had not been exposed to NE immediately prior to
187 adding to the attachment assay both showed a time dependent increase in attachment to the
188 Caco-2 cells, but at all time points more of the NE-treated spirochaetes were observed to be
189 attached (Fig. 4). The NE-exposed *B. pilosicoli* cells tended to be more clumped and tangled
190 than the non-exposed cells (Fig 4., panel D).

191 [Fig 4 about here]

192 Others have reported that NE can enhance attachment of bacteria species; for example,
193 exposure to 0.05 mM NE enhanced the attachment of *E. coli* O157:H7 to HeLa cells (Bansal
194 *et al.*, 2007). In future work it would be informative to investigate whether cultures of *B.*
195 *pilosicoli* exposed to NE cause increased cytopathic effects in the Caco-2 cells compared to
196 untreated cultures.

197

198 CONCLUSIONS

199 Exposure of *B. pilosicoli* to NE changed the behaviour of the spirochaete in a number of
200 ways that appear likely to increase its capacity to colonise the large intestine. It would be
201 instructive to test whether cultures that are exposed to NE *in vitro* do colonise better than
202 non-exposed cultures. Under natural conditions NE is present in the intestinal tract, and
203 elevated levels are likely to occur in periods of stress. It has recently been shown in
204 experimentally infected pigs that elevated plasma NE levels, such as those found in stressed
205 animals, were associated with increased faecal excretion of *Salmonella* Typhimurium
206 (Pullinger *et al.*, 2010b). Hence it seems likely that the *in vitro* observations may translate to
207 altered activity of the spirochaete *in vivo*.

208

209 The mechanisms involved in the change in *B. pilosicoli* behaviour require further study, but,
210 by analogy with other Gram-negative enteric pathogenic bacteria, they are likely to involve
211 mediation of iron acquisition to enhance growth, and/or alteration in gene expression by
212 activation of sensor kinases that may increase motility or other activities required for
213 colonisation (Hughes *et al.*, 2009; Pullinger *et al.*, 2010a; Reading *et al.*, 2010). Examination
214 of transcriptomics profiles of the spirochaete after addition of NE should help identify

215 potential pathways involved in the observed changes, and this work will be assisted by the
216 recent availability of a full genomic sequence for *B. pilosicoli* 95/1000 (Wanchanthuek *et al.*,
217 2010).

218

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222

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310

311

312 **Figure legends**

313

314 **Fig 1.** The effect of exposure to different concentrations of norepinephrine on the mean
315 number (\pm standard error) of *B. pilosicoli* 95/1000 cells grown in Kunkle's anaerobic broth
316 after four days incubation.

317

318 **Fig 2.** The effect of exposure to different concentrations of norepinephrine on the number
319 (mean \pm standard error) of *B. pilosicoli* 95/1000 cells entering capillary tubes containing 4%
320 mucin.

321

322 **Fig 3.** The effect of different norepinephrine concentrations in chemotaxis buffer on the
323 number (mean \pm standard error) of *B. pilosicoli* 95/1000 cells entering the buffer.

324

325 **Fig 4.** Attachment of *B. pilosicoli* 95/1000 to Caco-2 cell monolayers viewed with a scanning
326 electron microscope. Panels A and B show attachment following incubation for 2 hours.
327 Panels C and D show attachment following incubation for 6 hours. Panels A and C show
328 spirochaetes that were not exposed to norepinephrine; panels B and D show spirochaetes that
329 were exposed to 0.05 mM norepinephrine immediately before the assay.

330

331







