Significantly higher frequency of *Helicobacter suis* in patients with idiopathic parkinsonism than in control patients

C. Blaecher*,†, A. Smet*, B. Flahou*, F. Pasmans*, R. Ducatelle*, D. Taylor†,‡, C. Weller*, I. Bjarnason§, A. Charlett¶, A. J. Lawson**, R. J. Dobbs†,‡,§, S. M. Dobbs†,‡,§ & F. Haesebrouck*

*Faculty of Veterinary Medicine, Ghent University, Merelbeke, Belgium.
†Institute of Pharmaceutical Science, King’s College London, London, UK.
‡Department of Gastroenterology, King’s College Hospital, London, UK.
§Statistics Modelling and Economics Department of Centre for Infectious Disease Surveillance and Control, Public Health England, London, UK.
¶Gastrointestinal Bacteriology Reference Unit, Public Health England, London, UK.
**Gastrointestinal Bacteriology Reference Unit, Public Health England, London, UK.

Correspondence to:
Dr S. M. Dobbs, PO Box 42, 4th Floor Franklin-Wilkins Building, King’s College London, 150 Stamford Street, London SE1 9NH, UK.
E-mail: sylvia.dobbs@kcl.ac.uk

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**SUMMARY**

**Background**
There is increased proportional mortality from Parkinson’s disease amongst livestock farmers. The hypokinesia of Parkinson’s disease has been linked to *Helicobacter pylori*. *H. suis* is the most common zoonotic helicobacter in man.

**Aim**
To compare the frequency of *H. suis*, relative to *H. pylori*, in gastric biopsies of patients with idiopathic parkinsonism (IP) and controls from gastroenterology services.

**Methods**
DNA extracts, archived at a *Helicobacter* Reference Laboratory, from IP patient and gastroenterology service biopsies were examined anonymously for *H. suis*, using species-specific RT-PCR.

**Results**
Relative risk of having *H. suis* in 60 IP patients compared with 256 controls was 10 times greater than that of having *H. pylori*. In patients with IP and controls, respectively, frequencies of *H. suis* were 27 (exact binomial 95% C.I. 15, 38) and 2 (0, 3)%, and of *H. pylori*, 28 (17, 40) and 16 (12, 21)%. Excess of *H. suis* in IP held when only the antral or corporal biopsy was considered. Of 16 IP patients with *H. suis*, 11 were from 19 with proven *H. pylori* eradication, 3 from 17 pre-*H. pylori* eradication, 2 from 24 *H. pylori* culture/PCR-negative. Frequency was different between groups (*P* = 0.001), greatest where *H. pylori* had been eradicated. Even without known exposure to anti-*H. pylori* therapy, *H. suis* was more frequent in IP patients (5/41) than in controls (1/155) (*P* = 0.002). Partial multilocus sequence typing confirmed that strains from IP patients (6) and control (1) differed from RT-PCR standard strain.

**Conclusions**
Greater frequency of *H. suis* in idiopathic parkinsonism appears exaggerated following *H. pylori* eradication. Multilocus sequence testing comparison with porcine strains may clarify whether transmission is from pigs/porcine products or of human-adapted, *H. suis*-like, bacteria.

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INTRODUCTION

The broad brush of epidemiology has provided enigmatic aetiopathogenic clues in Parkinson’s disease (PD). It has been linked, albeit inconsistently, with rural living and farm experience. Such associations have long been explained away by putative exposure to agrochemicals. However, a mortality study in 26 US states found increased proportional mortality from PD among livestock, but not arable, farmers. From a total of 6 million death certificates, 267,479 decedents were classified as either crop or livestock farmers. Amongst white males, the proportional mortality from PD was significantly higher in livestock farmers than in nonfarmers, but lower in crop farmers. Zoonotic infections were considered as an explanation, but it was noted that the pesticides used in animal infestations are different from those used on crops. These effects were not replicated in the much smaller numbers of White female or of African American decedents.

The 1965 finding of prodromal peptic ulcers in PD paved the way to exploring any link between (the then undiscovered) *Helicobacter pylori* and PD. The 2012 Maastricht IV Consensus Report acknowledges interesting associations. In a randomised placebo-controlled trial, we found that biopsy-proven *H. pylori* eradication had differential effects on objective measures of PD facets: improvement in hypokinesia and worsening rigidity over the year post-eradication, both plateauing over the next year. Overall, there was clinically relevant improvement. This was independent of any (stable, long ½) anti-parkinsonian medication. Receipt of levodopa was an exclusion. Moreover, the effect on hypokinesia was indication-specific. A longitudinal observational study confirmed improvement in hypokinesia following *H. pylori* eradication. Anti-microbials for other indications had no such effect, but successive courses were associated with cumulative increase in rigidity.

Could, then, zoonotic-transmission of gastric non-*H. pylori* Helicobacter species (NHPH) contribute to PD? The term NHPH represents a group of closely related, but distinct, bacterial species found in different animal species, such as *H. suis* in pigs, and *H. felis*, *H. salomonis*, *H. bizzozeronii*, *H. heilmannii* sensu stricto, *H. cynogastricus* and *H. baculiformis* in cats and dogs. Although infection with these bacteria has been associated with human gastric disease in a substantial number of patients, human NHPH strains have been isolated on only three occasions. Subsequent identification to species level has revealed these to be *H. bizzozeronii* in two cases and *H. felis* in the other. Based on histopathology of gastric biopsy, the prevalence of NHPH in patients with gastric disease has been estimated at between 0.2% and 6%, depending on geographical distribution. Several studies have demonstrated that *H. suis* is the most frequent NHPH species in humans. However, its *in vitro* culture, starting from human gastric mucosa, has been unsuccessful so far, underlining the extreme fastidiousness of gastric NHPH in general and *H. suis* in particular.

We report the frequency of *H. suis* DNA in archived DNA extracts from gastric biopsies in a group of PD patients and a group of gastroenterology patients, as part of a service evaluation. The relative prevalence of *H. pylori* immunoblot seropositivity in people with and without PD (37% and 31% respectively) has been reported. Here, we standardise the frequency of *H. suis* positivity against that recorded for *H. pylori* (culture, or if negative, PCR), in order to compare the two sets of archived DNA extracts.

METHODS

Service evaluation

The term ‘*H. heilmannii*’ was commonly used to describe spiral gastric helicobacters seen on histopathology. The diagnostic service of the Gastrointestinal Reference Unit, Public Health England, for PCR detection of ‘*H. heilmannii*-like organisms’ in gastric biopsy material was re-evaluated, using anonymised archived DNA extracts. As *H. suis* is the commonest reported NHPH in humans and a species-specific assay is now available, it is a good starting point for evaluating whether NHPH cases are being missed. Interestingly, the original ‘*H. heilmannii*’ assay was set up in response to a case of a spiral helicobacter in PD.

Sourcing anonymised DNA extracts

The *Helicobacter* Reference Laboratory in the Gastrointestinal Bacteriology Reference Unit, Public Health England, held DNA from gastric biopsies, extracted (at time of receipt) over the last decade, stored at −80 °C. Biopsies had been received with request for *Helicobacter* culture (isolation/identification/anti-microbial susceptibility) and, if biopsy culture-negative, detection of *H. pylori*-specific DNA. Culture-negative biopsies had been tested using a PCR targeting 16S rRNA (primer pair HP1/HP2) and vacA (Vac3624F/Vac3853R) genes. To detect ‘*Helicobacter heilmannii*-like organisms’ a 16S
rDNA assay had been used.22 For the service evaluation, archived DNA extracts, identified only by their accession number, were couriered to the Laboratory of Veterinary Bacteriology and Mycology, Ghent University.

All extracts archived over this decade (paired antral and corporal biopsies) from 60 patients with ‘clinically definite’ idiopathic parkinsonism (IP),26 diagnosed at a National clinic, were retrieved. Clinically definite IP refers to any combination of three of the four cardinal features: resting tremor, rigidity, brady/hypokinesia, impaired postural reflexes. Alternatively sufficient is having two of the features, with one of first three asymmetrical (Responsiveness to a dopaminergic drug challenge was not a requirement). Details of anti-microbials used in any previous anti-*H. pylori* treatment (19/60) had been recorded on the request form.

DNA extracts from biopsies originating from English gastroenterological services, selected for attention to documenting any exposure to anti-*Helicobacter* therapy on the request form, were used as ‘controls’. This criterion yielded extracts from 256 patients, archived over a similar period (single biopsy on given occasion except in four, where two sites sampled). There had been previous exposure to anti-*Helicobacter* therapy in 101, no recorded exposure in the rest. No request form mentioned Parkinson’s disease, or any other issues outside the context of diagnostic endoscopy. Crude prevalence rate estimates for PD in 34 European studies range widely, from 65.6 to 12 500 per 100 00027: that is, 0–32 diagnosed cases would be expected in the gastroenterological services’ patient group, even if they had no particular predisposition.

Frequencies of *Helicobacter* species reported here are not construed as prevalence: there is selection in the gastroenterological aspects of PD. Any direct estimate of association between presence of *H. suis* and PD could be biased by cohort selection. We therefore obtain an estimate of the risk of *H. suis* in patients with IP compared with those from gastroenterological services by standardising for the relative risk of having *H. pylori*, and by setting *H. suis* frequency in context of exposure to anti-*Helicobacter* therapy. No analysis of the relationship of *H. suis* status to gastric symptoms was planned: the request form did not contain a checklist.

**H. suis** detection

The presence of *H. suis* DNA in the extracts was determined using a *H. suis*-specific quantitative real time (RT)-PCR, based on the *ureA* gene. For generating the standard, part of the *ureAB* gene cluster (1236 bp) from *H. suis* strain HS5 was amplified using primers U430F and U1735R, as described previously.14 The standard consisted of 10-fold dilutions, starting at 10^9 PCR ampli- cons, for each 9 µL of reaction mixture. One microlitre of extracted DNA template was added to 9 µL reaction mixture, consisting of 0.25 µL of both primers located within the 1236 bp fragment (to yield a 150 bp PCR product), 3.5 µL HPLC water and 5 µL SensiMix™ SYBR No-ROX (Bioline Reagents Ltd, London, UK). Sense primer was BF_HsuisF1: 5′-AAA ACA MAG GCG ATC GCC CTG TA-3′. Anti-sense primer was BF_HsuisR1: 5′-TTT CTT CGC CAG GTT CAA AGC G-3′. Annealing temperature was 62 °C. Both standards and samples were run in duplicate on a CFX96™ RT-PCR System with a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). To confirm the presence of *H. suis* DNA, all positive samples were sequenced.16 All 413 available extracts (including replicate biopsies at a given time and any follow-up biopsies) were assayed on two separate occasions to estimate between-assay agreement.

To demonstrate that the *H. suis* DNA in the biopsy extracts was different from the *H. suis* strain HS5 DNA used as standard in the RT-PCR, multilocus sequence typing (MLST) was performed with seven housekeeping genes, as previously described.28

**Statistical analysis**

Use of exact binomial confidence intervals provided an estimate of the uncertainty in estimates of proportions. The risk ratio, risk of having *H. suis* in IP patients to that in controls compared with relative risk of having *H. pylori*, was estimated from the paired data on the two species, using a conditional Poisson regression with robust standard errors.29

**RESULTS**

The distribution of age at time of first biopsy in controls [mean 52 (data interval 24, 81) years] encapsulated that in the IP patients [62 (45, 79) years]. There was no significant difference in the proportion of male patients: 57% of the IP patients were male (34/60), 47% (120/256) of the controls.

Overall agreement (99.3%) between the two *H. suis* RT-PCR assay runs on separate occasions was strong (n = 413, Kappa = 0.95, P < 0.001: null hypothesis of no agreement rejected) and there was no significant difference between the first and second run (exact McNemar’s test, ratio of paired proportions 1.11 [95% confidence
interval (CI) 0.99, 1.24], \( P = 0.25 \). Thirty-one of the 413 extracts were positive in either run, 28 in both. Nucleotide sequencing of positive samples, and subsequent Basic Local Alignment Search Tool (BLAST) analysis, revealed 95–100% homology with known \( H. suis \) strains.

The frequency of detection of \( H. suis \) and \( H. pylori \) in the extract(s), held in the archive from the 60 IP patients and 256 controls, is shown in Table 1. In patients biopsied on more than one occasion, only the first occasion is considered. Overall, \( H. suis \) DNA was present in 27 (binomial exact 95% CI 15, 38)% of the IP patients and in 2 (0, 3)% of controls. It was present in 18 (10, 30)% of antral biopsies and 13 (6, 25)% of corporal biopsies from IP patients. Thus, the excess \( H. suis \) frequency was not an artefact due to sampling both regions in IP, but only one region (46% antral, 1% corporal, 2% duodenal, rest ‘gastric’) in all but four controls (2 had antral and corporal biopsies, 1 antral and duodenal, in 1 both were labelled ‘gastric’).

### Table 1 | Frequency of \( H. suis \) contrasted with \( H. pylori \) in gastric biopsy DNA extracts from IP patients and controls with and without known exposure to anti-\( H. pylori \) therapy

| Frequency | IP patients % (no. with species/total) | Controls % (no. with species/total) |
|-----------|--------------------------------------|-----------------------------------|
| \( H. suis \) |                                      |                                   |
| Previous exposure to anti-\( H. pylori \) therapy | 58 (11/19)* | 3 (3/101) |
| No known exposure | 12 (5/41)* | 1 (1/155) |
| Total | 27 (16/60);† | 2 (4/256);‡ |
| \( H. pylori \) |                                      |                                   |
| Previous exposure to anti-\( H. pylori \) therapy | 0 (0/19) | 22 (22/101) |
| No known exposure | 41 (17/41) | 13 (20/155) |
| Total | 28 (17/60) | 16 (42/256) |

* Anti-microbial prescription was guided by in vitro sensitivities.† Of the 11 with \( H. suis \), 10 had received 1 week of amoxicillin, clarithromycin and proton pump inhibitor (PPI) (1 a further course of metronidazole, tetracycline, PPI and trimetoprim which when he remained urea breath test-positive) and 1 had received 1 week of clarithromycin, tetracycline and PPI.

Of 8 without \( H. suis \), 7 had received 1 week of amoxicillin, clarithromycin and PPI and 1 had received 1 week of clarithromycin, tetracycline and PPI.

† Three extracts also positive for \( H. pylori \).

‡ Mean age (gender) of the 16 IP patients with \( H. suis \) was 58 (range 47–68) years (10 male, 6 female), of the 4 controls, 54 (43–64) years (1 male, 3 female).

The relative risk of having \( H. suis \) in IP patients compared with controls was 9.9 times greater than that of having \( H. pylori \) (i.e. \([16/60]/(4/256))/(17/60)/(42/256)] = 17.07/1.73\). The 95% confidence interval (3.0, 32.7) did not include 1, indicating that the two relative risks are significantly different. This was despite a higher frequency of \( H. pylori \) in IP patients [28 (binomial exact 95% CI 17, 40)%] than in controls [16 (12, 21)%].

Of the 16 IP patients with \( H. suis \), 11 were from the 19 with proven \( H. pylori \) eradication, 3 from the 17 pre-\( H. pylori \) eradication and 2 from the 24 who were \( H. pylori \)-negative. Frequency of \( H. suis \) detection differed significantly among these three groups (Fisher’s exact test, \( P = 0.001 \)), being greatest in those who had undergone anti-\( H. pylori \) therapy than in the remainder. There was no significant difference in the anti-\( H. pylori \) regimen (clarithromycin/amoxicillin/proton pump inhibitor or other: footnote Table 1) between those with and without \( H. suis \) in its aftermath. Even in those without known previous exposure to anti-\( H. pylori \) therapy, \( H. suis \) was more frequent in IP patients (5/41) than in controls (1/155) \( (P = 0.002) \). The low frequency of \( H. suis \) in the 256 controls precluded estimation of any increased risk with exposure to anti-\( H. pylori \) therapy.

Partial MLST results for the \( H. suis \) DNA found in extracts from 6 IP patients and 1 control are shown in Table 2. The allele pattern in these samples differed from that in the HS5 DNA standard: that is, the sample results were not due to contamination. It was not possible to obtain complete MLST sequence types, due to interference from human DNA and the low amount of \( H. suis \) DNA found in some extracts.

None of the samples had been reported as positive for ‘Helicobacter heilmannii-like organisms’ on the original assay 16S rDNA assay.

### Table 2 | Partial \( H. suis \) MLST results on DNA extracts from gastric biopsies of IP patients and a control

| DNA extract | Allele no. |
|-------------|------------|
| atpA | efp | mutY | ppa | trpC | ureAB | yphC |
| IP patient 1 | 4 | 1 | 11 |
| IP patient 2 | 1 | 1 |
| IP patient 3 | 1 | 1 |
| IP patient 4 | 1 | 1 | 11 |
| IP patient 5 | 5 | 1 | 1 |
| IP patient 6 | 1 | 5 |
| Control patient | 1 | 1 | 1 |
| HS5* | 4 | 2 | 4 | 1 | 1 | 1 |

* Positive control in the quantitative RT-PCR assays.
DISCUSSION

Relative frequency of *H. suis* in IP
The frequency of finding *H. suis* DNA in extracts from gastric biopsies in a well-defined disease state, clinically definite IP, contrasts sharply with the apparently ‘sporadic’ nature in patients from gastroenterological services undergoing diagnostic endoscopy. The statistical significance of the finding withstood standardisation for the relative frequency of *H. pylori* in the two patient groups. However, defining the strength of association between presence of *H. suis* DNA in gastric biopsies and PD will clearly require representative cohorts of IP probands and controls. As human infection with NHPH is characteristically sparse and patchy, we may be underestimating the true frequency of *H. suis* in both patient groups. There are no previous studies of the frequency of NHPH in PD for comparison.

Proposed pathophysiological role of *Helicobacter* species in IP
The concept of ‘brain-altering’ remote infections is receiving increasing attention. Neuro-inflammation in IP may not merely be the adverse effect of microglial scavenging of degenerating neurons and reaction to aberrant protein. If PD is driven by systemic immuno-inflammatory processes, intervention against them, or their driving forces, could modify its course.

A U-turn in brady/hypokinesia and weight gain was described after eradicating a spiral *Helicobacter*, associated with antral gastritis, in a cachectic IP patient, who had been wheelchair-bound without assistance for over a year. The aetiopathogenic significance of zoonotic helicobacters in IP remains to be explored. Finding clinical correlates (e.g. epidemiological, such as mortality, and pathophysiological, such as circulating inflammatory markers) would suggest that *H. suis* DNA in gastric mucosa is of significance in IP.

Autoimmunity is suggested as the mechanism of the effect of *H. pylori* eradication on hypokinesia. Response appears unrelated to infection load. Poor response is associated with anti-nuclear antibody sero-positivity. There are HLA-DR risk loci for PD. If NHPH eradication has a similar effect, then the immune hypothesis could move to a pattern recognition response rather than classical HLA-restricted autoimmunity.

Optimising diagnosis of human *H. suis* infection
Diagnosing NHPH in IP is problematic. Our experience is that the urea breath test is usually negative, except where *H. pylori* co-exists. A serological test for *H. suis* would provide a useful screen for infection, or any memory of it, in potentially high-risk patient or occupational groups, but none is currently available. The 16S rDNA assay for ‘*Helicobacter heilmanni*’-like organisms did not detect the *H. suis* found on RT-PCR. It may, in part, be a question of sensitivity. *Helicobacter heilmanni*-like DNA has been detected in a urea breath test-positive case: re-evaluation by the methodology used here showed this to be *H. suis*. There appears to be advantage in taking more than one biopsy to determine NHPH status by molecular microbiology. Low infection load will hamper histological confirmation. Sensitivity and specificity of the RT-PCR will need to be determined for introduction into routine clinical use. This is not as problematic for *H. suis* as for other NHPH, since it is the only gastric *Helicobacter* isolated from pigs in Europe, where infection is very common and load high. The gold standard of seeing spiral gastric helicobacters, in a histological section adjacent to the biopsy for DNA extraction, can be applied.

Greater frequency of *H. suis* in IP where *H. pylori* has been eradicated points to need for post-treatment endoscopic biopsy. New infection in a particularly susceptible host cannot be excluded, although adult transmission of another gastric helicobacter, *H. pylori*, is unusual. It is likely that the IP patients were co-infected with *H. pylori* and *H. suis* prior to eradicating *H. pylori*, and *H. suis* filled the niche afterwards. Indeed, susceptibility testing of isolates from sows suggests relative intrinsic insensitivity to amoxicillin and metronidazole, greater intrinsic susceptibility to tetracycline. Current use of tetracycline in first-line treatment strategies is limited. Information on *in vitro* anti-microbial susceptibility of *H. suis* in man is needed.

Transmission of *H. suis*
Contact with pigs is a risk factor for human gastric *H. suis* infection. It was demonstrated, using MLST, that a *H. suis* strain from the stomach of a pig veterinarian with gastric complaints was closely related to porcine strains. This illustrates the zoonotic potential of this species: direct contact with pigs can be a source for human *H. suis* infection. Moreover, *H. suis* can be present, and survive, in minced pork: raw or undercooked porcine products might be another source of infection. Complete MLST sequence types were not obtained for any of the *H. suis* strains from patients, precluding comparison with sequence types present in pigs. Although all alleles of the housekeeping genes detected
A natural progression of the work is to examine the archived samples for zoonotic helicobacters commonly associated with cats and dogs.

**AUTHORSHIP**

Guarantor of the article: Dr Sylvia Dobbs.

Author contributions: RJD, SMD, IB and AL requested FH’s expertise, in re-evaluating the NHPH assay used at Public Health England. FH, RJD and SMD were joint principal investigators. CB & AS contributed equally. CB, AS, BF, AL performed the laboratory work. CB, AS, AL, FH, SMD, RJD, AC collected and analysed the data. AS, AL, AC, RJD, SMD, FH designed the study. CB, BF, FR, RD, DT, CW, IB contributed to the design. CB, AS, FH, IB, AC, SMD, RJD wrote the paper. All authors have approved the final version of the manuscript.

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