1 *Executive Summary*

This study has conducted developmental work to improve the methodology for isolation of *Helicobacter* species, including *H. pylori.* The study developed improved protocols for the collection, processing and culture of samples. The survival and recovery of *H. pylori* subjected to stress conditions, including limited nutrients and low temperature, was investigated.

The study involved a sampling programme of three phases. Phase one was a survey of domestic properties. Samples were collected from a total of 62 properties recruited from 13 different areas within Greater London; 41 from the Northwest London area, 16 from North London, one from each of East, West and South London and no information was obtained from two properties. They consisted of 56 biofilm and 62 water samples. Phase two involved the recruitment of samples from properties that were Polymerase Chain Reaction (PCR)-positive for Helicobacter by at least one of the three PCR assays (HGS16S targeting the 16S rRNA *Helicobacter* genus-specific gene, HPVacA and 16SHP targeting the VacA and 16SrRNA *H. pylori* species-specific genes respectively) in sampling phase one. Samples were collected from nine properties; they consisted of both biofilm and water samples. A modified protocol was tested against samples from domestic properties collected in phase three. Both biofilm and water samples were collected from a total of 27 properties; eight of which were properties that had provided PCR positive samples in the first round. Seven properties were included in each sampling phase. In addition, two water effluent samples; primary and final effluent, were supplied by the sewage treatment works.

1.1 Development of a protocol for culture of *H. pylori* from water and biofilm samples

A range of parameters was evaluated in order to improve the detection, isolation and recovery of *H. pylori* from domestic water and biofilm samples. This included

assessment of media containing different levels of nutrients and selective agents and the development of enrichment broth for the primary isolation of *H. pylori*.

1.2 Evaluation of solid culture media

Four solid culture media (Columbia Blood Agar, Columbia Blood Agar containing twice the concentration of Dent's antibiotic supplement, Degnan agar and Half strength *H. pylori* special peptone agar) were evaluated for their ability to recover helicobacter from Maximum recovery diluent (MRD) and *H. pylori* that had undergone nutrient and temperature shock by suspension in sterile tap water.

Overall, CBA medium allowed the best recovery of *H. pylori* after suspension in MRD for up to 24 hours, both in terms of speed of growth and colony counts. The Degnan agar medium gave the least satisfactory results. There were considerable variations in growth characteristics between strains. Nevertheless, all strains of *H. pylori* showed poor recovery after suspension in filtered tap water. The ability to recover each strain decreased significantly after 24h incubation in tap water. For all strains, the highest colony counts from filter-sterilized tap water were observed on CBA, although a longer incubation of 72h was required to visualise colonies.

1.3 Evaluation of enrichment broths

An enrichment step is beneficial for increasing the numbers of *H. pylori* but the growth of background contamination needs to be controlled. Enrichment with four blood-free broth media (MRD, Full Strength special peptone (FSPB), Half strength special peptone broth (ASPB), Brucella broth with 0.1% β cyclodextrin (BBβC)) were tested. None of the broths enriched the growth of *H. pylori*. However, colonies were visible for longer on the plates when they were incubated in BBβC. The reduction in colony forming units on CBA plates from 24 and 48h broth cultures suggests that there is cell death or conversion of cells to a non-culturable (VNC) state. Exclusion of antibiotics from the broth did not significantly improve enrichment.

The ability to enrich *H. pylori* growth with two blood-containing liquid media (Brucella broth with 1% yeast extract (BBYE), Brucella broth with 1% yeast extract and 7% defibrinated horse blood (BBYEB), Mueller-Hinton broth with 1% yeast extract (MHYE), Mueller-Hinton broth with 1% yeast extract and 7% defibrinated horse blood (MHYEB)) was also evaluated. The results demonstrated the potential of blood-containing broths to enrich *H. pylori* growth. The period of incubation required and the level of enrichment are strain-dependent. Additionally prolonged incubation of broths lead to overgrowth of bacterial contaminants.

1.4 Evaluation of methods for initial sample processing

Both physical and chemical methods of sample processing were explored to reduce levels of contaminating microflora and to concentrate *H. pylori*. These included double filtration, differential centrifugation, acid treatment, use of antibiotic supplements and the application of Immunomagnetic Separation (IMS).

Findings from experiments, which explored different filter pore sizes (0.65µm, 0.45 µm and 0.2 µm) for their capacity to trap *H. pylori* cells indicated that *H. pylori* cells could not penetrate membranes with pore sizes of 0.45 µm or 0.2 µm. Different strains of *H. pylori* show different levels of acid tolerance. However, acid treatment reduced the amount and mixture of bacterial contamination. Meanwhile, higher numbers of *H. pylori* colonies were recorded when levels of bacterial contaminants were reduced.

A combination of differential centrifugation and double filtration revealed that despite a reduction in bacterial contamination from water samples, the proportion of the *H. pylori* cells lost during the first lower speed centrifugation outweighed any benefit of its application.

The acid treatment step showed promise for reducing the level of background contamination, without affecting the recovery of *H. pylori*. The highest rate of *H.*

pylori recovery was from the acid treated samples plated on Dent's medium, where the lowest levels of bacterial contamination were observed.

Concentrating *H. pylori* cells from a sample by magnetic beads coated with specific (anti-*H. pylori*) antibody to capture *H. pylori* cells was explored. This approach reduced the recovery of *H. pylori* and the method was not pursued further. .

1.5 Evaluation of a preliminary culture protocol

Testing the initial protocol developed during this study with samples collected at phase two showed that contamination was a consistent problem on the majority of the culture plates. This was more apparent on plates derived from water samples than those from biofilm samples. Moreover, for the biofilm samples, more than 55% of the Dent's, CBA2D and Degnan plates inoculated with broth and antibiotics, did not yield any bacterial growth, regardless of the incubation time. This is surprising and may be explained by the fact that biofilm was not recovered on the swab when sampling the toilet cistern.

1.6 Application of culture and molecular protocols to survey domestic properties

Contamination levels were too high in some samples collected in phase two, in particular the water samples. Therefore, the possibility that *H. pylori* are present should not be excluded. However, *H. pylori* were not isolated on any of the plates where contamination was absent or only moderate. These results may indicate that *H. pylori* are not present, not culturable from these samples, or that the cells have entered a viable but non-cultivable state. Alternatively, *H. pylori* may be present at a level that is below the threshold of sensitivity for the methods developed.

A modified protocol for isolating *H. pylori* from domestic water samples was applied to samples collected in phase three. This protocol included two extra broths, one with antibiotics and one without, for samples that had not undergone

acid treatment. No *H. pylori* have been cultured in 54 samples from 27 properties from this round of sampling. Observations indicate that contaminating growth continues to be a problem. This study has highlighted the limitations of a wider application of this protocol developed during the course of the study.

PCR results indicated that 15/118 (12.7%) of samples collected from phase one, were weakly positive by at least one PCR assay. Two of the 15 were positive for all three assays (Logan *et al*. 2000, Ho *et al*. 1991 and Chisholm *et al*. 2001) although the Ho *et al*. 1991 and the Chisholm *et al.* 2001 assays were very faint positives. One of the 15 was positive by the Logan *et al*. 2000 and the Ho *et al*. 1991 assay and the remaining 12 samples were positive by the Logan *et al*. 2000 assay alone. All 18 samples collected during phas two were PCR-negative, while 11/54 samples from phase three were positive by at least one PCR assay.

No *H. pylori* were recovered in culture from samples collected from phase 2. All samples were PCR negative. This was unexpected as the samples were from previously PCR positive properties, including one property that had a biofilm sample positive in all three PCR assays. This may suggest that the presence of *H. pylori* in domestic properties is a transient occurrence. It is possible that the survival of helicobacter is poor during the summer months and / or there may be a higher disinfectant residual chlorine/ monochloramine in the mains water during the summer.

1.7 Risks to people consuming mains drinking water

The study has demonstrated that although *H. pylori* can be detected by molecular methods in drinking water the organism has not been isolated, despite extensive cultural investigation. Even if viable organisms were present in such low numbers, that their successful culture using the presently available culture protocols remains unresolved because of heavy overgrowths of other microorganisms in the water and biofilms. It is most likely that these organisms

are dead or in a viable but non-culturable state. This is because there is considerable evidence for the poor survival of *H. pylori* in water, evidence that disinfection used in treatment and as a residual should prevent its survival and lack of clear evidence that these organisms are viable but non-cultivable. While it remains possible that occasional contamination of mains water with *H. pylori* could occur the surveillance systems would not be able to detect an outbreak of *H. pylori* gastritis. It remains likely that the predominant mode of transmission of *H. pylori* in the UK is by person-to-person transmission in childhood.