Further analysis of the incidence of *Mycobacterium avium* Complex (MAC) in drinking water supplies (Including the detection of *Helicobacter pylori* in water and biofilm samples)

DWI/70/2/146 Extension

Analysis of water samples for MAP by direct IS900 PCR

A research report from the Health Protection Agency to the Drinking Water Inspectorate

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INTRODUCTION

Mycobacterium avium subsp. *paratuberculosis* (MAP) is a putative cause of Crohn's disease, a chronic inflammatory disease of the gastrointestinal tract of humans. The association is poorly understood, and based largely on clinical similarity between Crohn's disease and Johne's disease, a chronic, contagious and lethal disease that effects a wide range of livestock and domestic and wild animals, of which MAP is a known etiological agent. The presence of MAP in the faecal matter of clinically and subclinically infected cattle is well established (Whitlock *et al*., 1985 and Stabel, 1998). The entry of MAP into the water system is plausible, for example in agricultural run-off from the land into rivers. Given the zoonotic potential of MAP it is important to identify the incidence and extent of MAP in the environment and assess the exposure and risk to humans.

Concern has been raised because of some studies in which MAP DNA has been detected in human biopsy samples taken from Crohn's patients. For example, MAP DNA was detected in 65-95% of samples taken from the guts of patients with Crohn's (Sanderson *et al*., 1992 and Bull *et al*., 2003). Likewise the direct detection of MAP in samples may be due to the presence of DNA from dead organisms and not necessarily live organisms. It is also possible that components of dead mycobacteria may be relevant in acting as a trigger for Crohn's.

Recent studies report the presence of both MAP DNA and viable MAP in bulk tank and pasteurised milk (Pillai and Jayarao, 2002, Stabel *et al*., 2002, Cousins *et al*., 1999). The reported detection rates of MAP directly from samples (by molecular methods) compared to culture of viable MAP from samples were apparently higher. This has caused debate because it is hard to establish whether or not the low culture

rates are a reflection of the extremely slow growth rate and fastidious nutritional requirements of the organism.

In addition to the presence of MAP in milk concern has been expressed at the potential contamination of drinking water. In a recent study (Whale *et al*., 2004), no MAP was cultured from a range of samples collected from domestic water systems, including mains drinking water. This study demonstrated the problems associated with attempts to isolate MAP from environmental samples, particularly contamination and competition from rapid growing and more abundant mycobacteria species. For these reasons we proposed to use direct molecular detection methods. The most established methodology is detection of the insertion sequence 900 (IS900) by a polymerase chain reaction (PCR)-based assay. IS900 is a 1,451 bp element inserted into the MAP genome at 14-18 conserved loci (Bull *et al*., 2003). A number of protocols have been developed and commercial kits are available with varying performance (Pilliai and Jayarao, 2002). An optimised and specific nested IS900 PCR (in which the products of a primary PCR reaction serve as the template for a second PCR) as described by Bull *et al*. (2003) was adopted in this study due to its increased sensitivity over conventional IS900 PCR. The feasibility of alternative approaches for MAP detection such as immunomagnetic separation (IMS) or long term culture prior to PCR analysis was also examined.

This aim of the study was to retrospectively analyse the samples collected in the previous DWI study (listed in appendix) using IS900 analysis. Whilst we have had success using a cheap and rapid crude DNA extraction, others have advocated the importance of mechanical disruption, the use of Proteinase K and DNA purification (Pilliai and Jayarao, 2002, Bull *et al.,* 2003 and Irene grant, personal communication).

MATERIALS AND METHODS

Sample collection

Sampling methods were as detailed in the prior report to the DWI. The samples used in this experiment were 1ml aliquots of those samples, stored at the MRU at –20^{o}C. Details of the sample sites and types are detailed in the appendix and in greater detail in the previous report. Approximately 5ml of each sample remain archived at HPA, Colindale. DNA from the 1ml aliquots of each sample stored at the MRU were extracted and assayed using the IS900 PCR to directly detect MAP DNA in the samples as follows:

Three different methodologies were used to extract DNA directly from the water samples in this study with the quick extraction compared to either the matrix or the ribolyser methods.

Quick DNA extraction

500µl sample was incubated in an equal volume of chloroform for 20 mins at 80^{o}C. The reaction mix was then cooled on ice for 5 mins, spun at 12,000g for 5 mins. The upper aqueous layer containing the extracted DNA was removed and stored at –20^{o}C prior to use.

Matrix DNA extraction

500µl sample was spun at 20,800g for 30 mins, and the pellet resuspended in 100µl supernatant. The spun sample was incubated with 200µl stirred matrix (BioRad) at 56^{o}C for 30mins. The reaction mix was then vortexed for 10 seconds before incubation at 100^{o}C for 10 mins. The reaction mix was then spun at 20,800g for 5 mins and stored at –20^{o}C.

Ribolyser DNA extraction

500µl sample was incubated for 30mins with 10mg/ml proteinase K at 37^{o}C. After proteinase K treatment, samples were transferred to labelled FastRNA lysing matrix B blue-capped tubes (Qbiogene-Alexis) and placed in a Hybaid Ribolyser for 45sec at 6.5m/s. After mechanical disruption the tubes were cooled on ice for 15mins. 700µl of phenol:chloroform:isoamylalcohol (25:24:1), pH 8.0 (sigma) was subsequently added and the resultant mix vortexed for 1 min. Tubes were then centrifuged 12,000g for 10 mins. The upper aqueous layer was transferred to a fresh 1.5ml eppendorff tube containing 400µl isopropanol to precipitate DNA. The samples were incubated at –20^{o}C for 30 mins and DNA was recovered by centrifugation at 12,000g for 15mins. The pellet was washed with 70% ethanol and respun at 12,000g for 15mins. The DNA pellet was allowed to air dry, resuspended in 50µl sterile water and stored at –20^{o}C.

IS900 PCR

IS900 nested PCR was performed to distinguish MAP isolates (IS900 positive) from all other *M. avium* subspecies (IS900 negative) e.g. *M. avium* Complex (MAC) and *M. avium intracellulare* (MAI). It is also capable of detecting MAP DNA in samples. Each PCR run included a MAP PCR positive control and *M.avium* negative control in addition to PCR negative controls.

The first round of PCR used the following primers:

TJ1: 5’ GCT GAT CGC CTT GCT CAT 3’
TJ2: 5’ CGG GAG TTT GGT AGC CAG TA 3’

and the cycle conditions

94^{o}C for 10min x1		
94^{o}C for 30min		
58^{o}C for min	}30 cycles	
72^{o}C for 3 min		
72^{o}C for 7min x1		

The second round (nesting) of PCR uses the primers

TJ3: 5' CAG CGG CTG CTT TAT ATT CC 3'
TJ4: 5' GGC ACG GCT CTT GTT GTA 3'

and identical cycle conditions. In order that amplicon contamination was avoided nesting was performed in the template tamer and the TJ1 & 2 and TJ3 & 4 master mixes were prepared simultaneously.

Hot star taq	2U
Buffer with $MgCl_2$	1x
Primer 1	2µM
Primer 2	2µM
dNTP's	100µM
DMSO	10%
Water	up to 50µl
Sample	2.5µl

From each primary amplification, 2.5µl was used as the template for the second round of PCR.

The final product (5µl) was visualised on a 1.5% agarose gel. The IS900 nested product was 294bp in size.

Prolonged incubation:

A random group of 59 samples was selected plus the water meter sample (11090) from which the 'IS900-like' organism was cultured. 1ml of each sample was decontaminated with 0.7% CPC using the methods previously described (Whale *et al.*, 2004). The decontaminated samples were then inoculated into MB BacT bottles along with antibiotics, 0.5ml Egg yolk emulsion and Mycobactin J. (Performed on 28th November 2003).

RESULTS

The sensitivity of the nested IS900 assay was established in spiking experiments in both sterile water and a shower water sample (99/11043). Because of the limited time frame of the study and the long culture times required for MAP (8-16 weeks), it was not possible to determine the sensitivity of the PCR in terms of CFU/ml. As a result, the sensitivity is expressed in terms of amount of detectable DNA, based on spectroscopic measurement of the DNA concentration present in neat, control extractions and back calculation using the dilution factor.

The data in Table 1 shows that the IS900 protocol employed in this study is able to detect DNA present at a concentration of 20pg/ml in both sterile and shower sample water.

Table 1: Sensitivity of IS900 PCR assay

Dilution	IS900 PCR	
	Sterile water	Shower water
Neat (200 µg ml^{-1})	+	+
10^{-1}	+	+
10^{-2}	+	+
10^{-3}	+	+
10^{-4}	+	+
10^{-5}	+	+
10^{-6}	+	+
10^{-7}	+	+
10^{-8}	-	-
10^{-9}	-	-
10^{-10}	-	-
-ve Control (MAC)	-	-
+ve Control	+	+

Two different MAP strains were used as positive controls in this study: MAP strain NCTC 7992 (which is equivalent to ATCC 199698) and a bovine MAP strain supplied by Queens University Belfast.

Table 2 details the IS900 PCR results obtained from each water sample, using different DNA extraction techniques. The Matrix and Ribolyser extraction techniques were tested on independent randomly selected subsets of the samples for efficiency. Each sample was analysed in duplicate, with one sample spiked with a 1in 100 dilution of neat control MAP DNA in order to establish if any PCR inhibition was taking place.

Table 2 - = PCR negative; + =PCR positive; blank = DNA extraction method not done

Sample number	IS900 PCR			
	Quick extraction	Matrix extraction	Ribolyser extraction	Spiked sample
99/11111	-		-	+
99/11112	-	-		+
99/11113	-			+
99/11114	-			+
99/11115	-			+
99/11116	-	-		+
99/11117	-			+
99/11118	-			+
99/11001	-		-	+
99/11002	-			+
99/11003	-	-		+
99/11004	-			+
99/11005	-			+
99/11006	-	-		+
99/11007	-			+
99/11008	-			+
99/11009	-			+
99/11010	-			+
99/11011	-			+
99/11012	-			+
99/11013	-			+
99/11014	-		-	+
99/11015	-			+
99/11019	-			+
99/11020	-			+
99/11021	-			+
99/11022	-		-	+
99/11023	-	-		+
99/11024	-			+
99/11025	-			+
99/11026	-			+
99/11027	-		-	+
99/11028	-			+
99/11029	-			+
99/11030	-	-		+
99/11031	-			+
99/11032	-			+

Sample number	IS900 PCR			
	Quick extraction	Matrix extraction	Ribolyser extraction	Spiked sample
99/11033	-	-		+
99/11034	-			+
99/11035	-		-	+
99/11036	-			+
99/11037	-			+
99/11038	-	-		+
99/11039	-		-	+
99/11040	-			+
99/11041	-			+
99/11042	-			+
99/11043	-	-		+
99/11044	-			+
99/11045	-			+
99/11046	-		-	+
99/11047	-			+
99/11048	-	-		+
99/11049	-			+
99/11050	-			+
99/11051	-		-	+
99/11052	-			+
99/11053	-			+
99/11054	-			+
99/11055	-			+
99/11056	-			+
99/11057	-			+
99/11058	-	-		+
99/11059	-	-		+
99/11060	-			+
99/11061	-			+
99/11062	-			+
99/11063	-			+
99/11064	-		-	+
99/11065	-			+
99/11066	-			+
99/11067	-			+
99/11068	-	-		+
99/11069	-			+
99/11070	-			+
99/11071	-			+
99/11072	-	-		+
99/11073	-			+
99/11074	-			+
99/11075	-			+
99/11076	-		-	+
99/11077	-			+
99/11078	-		-	+
99/11079	-	-		+
99/11080	-			+
99/11081	-	-		+
99/11082	-	-		+

Sample number	IS900 PCR			
	Quick extraction	Matrix extraction	Ribolyser extraction	Spiked sample
99/11083	-			+
99/11084	-			+
99/11085	-			+
99/11086	-			+
99/11087	-			+
99/11088	-		-	+
99/11089	-			+
99/11090	-			+
WM 236	-	-		+
WM 237	-			+
WM 238	-			+
WM 239	-			+
WM 240	-			+
WM 241	-			+
WM 243	-			+
WM 244	-			+
WM 245	-			+
WM 246	-			+
WM 247	-	-		+
WM 248	-		-	+
WM 249	-		-	+
WM 250	-			+
WM 251	-		-	+
WM 252	-	-		+
WM 253	-			+
WM 254	-			+
WM 255	-			+
WM 256	-			+
WM 257	-	-		+
WM 258	-			+
WM 259	-			+
WM 260	-		-	+
WM 261	-			+
WM 262	-			+
WM 263	-			+
WM 264	-			+
WM 265	-			+
WM 266	-			+
WM 267	-	-		+
WM 268	-			+
WM 269	-			+
WM 270	-			+
WM 271	-			+
WM 328	-			+
WM 329	-			+
WM 330	-			+
WM 331	-		-	+
WM 332	-			+
WM 333	-	-		+
WM 334	-			+

Sample number	IS900 PCR			
	Quick extraction	Matrix extraction	Ribolyser extraction	Spiked sample
WM 335	-			+
WM 336	-			+
WM 337	-			+
WM 338	-			+
WM 339	-			+
WM 340	-		-	+
WM 341	-	-		+
WM 342	-			+
WM 344	-		-	+
WM 345	-			+
WM 346	-	-		+
WM 347	-			+
WM 348	-		-	+
WM 349	-			+
WM 350	-			+
WM 351	-			+
WM 352	-			+
WM 353	-			+
WM 354	-	-		+
WM 355	-		-	+
WM 356	-			+
WM 357	-			+
WM 358	-			+
WM 359	-	-		+
WM 360	-			+
WM 361	-			+
WM 362	-			+
WM 363	-			+
WM 364	-			+
WM 365	-			+
WM 366	-			+
WM 367	-	-		+
WM 368	-			+
WM 369	-			+
WM 370	-			+
WM 371	-			+
WM 372	-			+
WM 373	-			+
WM 374	-		-	+
WM 375	-			+
WM 376	-		-	+
WM 377	-			+
WM 378	-			+
WM 379	-			+
WM 380	-			+
WM 381	-			+
WM 382	-			+
WM 383	-			+
WM 384	-	-		+
WM 385	-			+

DISCUSSION

The overall sensitivity of the IS900 PCR assay is good, detecting 20 pg/ml of MAP DNA in sterile and shower water. Nevertheless on direct PCR none of the water samples was positive for MAP DNA. This was not due to the presence of PCR inhibitors as none of the spiking experiments demonstrated the presence of inhibition i.e all the DNA purification methods removed or neutralised any inhibitors present.

We did not perform IMS on these samples following advice taken from Dr Irene Grant at Queen's University Belfast. She emphasised the importance of treating samples with proteinase K and using a ribolyser approach to pre-prepare the sample and maximise the DNA that could be obtained for analysis. She kindly provided a protocol for this, which was incorporated and used above in place of the aims approach. From our analysis of the sensitivity of culture and other methods for MAC it would appear that MAP organisms are probably present (if at all) in concentrations that are below the thresholds for IMS or PCR (between 2 and 10 CFU/ml). Most IMS antibodies are polyclonal and may lack the necessary specificity to accurately distinguish MAP.

One possible solution is to culture samples in MB BacT for a prolonged period accepting that other mycobacteria and contamination may outgrow MAP. The hypothesis would be that there might be a small but sufficient increase in MAP biomass that would be enough for molecular detection i.e. attempts to detect DNA of MAP in a mixed culture. A liquid culture system was chosen as it gave the best overall growth for mycobacteria. In a recent study Bull *et al*. (2003) cultured biopsy samples for 14-88 weeks to yield MAP DNA signals. In our previous study, cultures were incubated for 16 weeks in line with previously developed protocols. This may not allow sufficient time particularly if one considers that there may be only a single bacillus in a sample. A retrospective analysis from the MB BacT cultures was not

possible as these were subcultured onto HEYM media to allow for phenotypic identification. For these reasons a random collection of 60 samples was set up for prolonged incubation for final analysis in 2004.

FINAL RESULTS – FEBRUARY 2005

Fifty of 60 original cultures growing in a volume of 10 ml of 7H9 medium (BacT Alert media, Biomerieux) were suitable for examination. Cultures were centrifuged and the resulting bacterial pellet resuspended and heat-treated with chloroform and the DNA extracted.

Two positive controls of MAP were resuspended in media and DNA extracted in the same way as the test cultures. From each sample 1 ul of DNA extract was taken for DNA amplification using a nested PCR to detect IS 900 using primers TJ1 and TJ2 for the first round, and TJ3 and TJ4 for the second nested PCR. Reaction conditions were as described and used previously. The PCR products were analysed on 3% (w/v) agarose gels.

Whilst both the positive controls did amplify to give a product of the required size, neither the negative control nor any of the samples tested gave a product of any description.

CONCLUSIONS and FURTHER WORK NEEDED:

Many unanswered questions remain with MAP:

- there is no proven link to human disease
- there have been few studies of healthy humans and it is uncertain if this organism is part of a regular transitory flora in the gut
- there continues to be uncertainty of the most optimal methods and the balance between false positives and sensitivity.

The close relationship of Johne's disease and MAP requires further exploration of how MAP might survive in the environment. In particular there is a need to assess whether mycobacteria in general and MAP/MAC in particular are able to survive in a quasi-symbiotic relationship with amoebae, algae and other comparable life forms using *in vitro* models. If they do then most of the approaches taken to prove that viable MAP is or is not present in water would not be valid; correspondingly if we could understand how MAP survives in the environment this would offer alternative measures to ensure successful eradication.

In conclusion the negative results from the direct detection methods used here does not rule out presence of organism, just exemplifies the fact that MAP is hard to culture, hard to detect, and probably present in very small numbers (less than 1 CFU/ml).

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APPENDIX

Sample number	Sample type	Sample location
99/11111	10 litre	EL1 Bathroom cold tap
99/11112	10 litre	EL1 Kitchen cold tap
99/11113	10 litre	EL1 Shower water
99/11114	2.5 litre	EL1 Kitchen hot water
99/11115	Biofilm swab	EL1 Shower
99/11116	Biofilm swab	EL1 WC
99/11117	Net deposit	EL1 Kitchen cold tap
99/11118	2.5 litre	EL1 Bathroom hot tap
99/11001	10 litre	EL2 Mains water supply school office
99/11002	10 litre	EL2 Header tank water from school office tank
99/11003	2.5 litre	EL2 Hot water, furthest from boiler
99/11004	2.5 litre	EL2 Hot water, closest to boiler, office tap
99/11005	Net deposit	EL2 School office tap
99/11006	Biofilm swab	EL2 Header tank
99/11007	Biofilm swab	EL2 WC
99/11008	10 litre	EL3 Bathroom cold tap, furthest from inlet
99/11009	10 litre	EL3 Shower water
99/11010	10 litre	EL3 Kitchen tap, mains supply
99/11011	2.5 litre	EL3 Bathroom hot tap, furthest from boiler
99/11012	2.5 litre	EL3 Bathroom hot tap, closest to boiler
99/11013	Biofilm swab	EL3 Shower hose
99/11014	Biofilm swab	EL3 toilet cistern
99/11015	Net deposit	EL3 Kitchen tap, mains water
99/11019	10 litre	Hydrant outside 15 Mulberry, E18
99/11020	10 litre	Hydrant outside 21 Laura close, E11
99/11021	10 litre	Hydrant outside 45 Arundel close, E11
99/11022	Net deposit	Hydrant outside 15 Mulberry, E18
99/11023	Net deposit	Hydrant outside 21 Laura close, E11
99/11024	Net deposit	Hydrant outside 45 Arundel close, E11
99/11025	10 litre	NW1 Kitchen cold tap, mains water
99/11026	10 litre	NW1 Header tank water
99/11027	10 litre	NW1 Shower water
99/11028	2.5 litre	NW1 Shower room hot tap, furthest from boiler
99/11029	2.5 litre	NW1 Kitchen hot water, closest to boiler
99/11030	Net deposit	NW1 Kitchen tap, mains supply
99/11031	Biofilm swab	NW1 WC cistern
99/11032	Biofilm swab	NW1 Shower head
99/11033	Biofilm swab	NW1 Header tank
99/11034	2.5 litre	NW 2 Hot tap water, furthest from boiler
99/11035	2.5 litre	NW2 Hot tap water, closest to boiler
99/11036	10 litre	NW2 Boys toilets tap, Header tank water
99/11037	10 litre	NW2 Shower water
99/11038	10 litre	NW2 Staff room tap, mains supply
99/11039	2.5 litre	NW3 Hot water tap, closest to boiler
99/11040	2.5 litre	NW3 Hot water tap, furthest to boiler
99/11041	10 litre	NW3 Bathroom cold water, header tank
99/11042	10 litre	NW3 Kitchen mains supply
99/11043	10 litre	NW3 Shower water
99/11044	Biofilm swab	NW2 WC cistern

Sample number	Sample type	Sample location
99/11045	Biofilm swab	NW2 Header tank
99/11046	Biofilm swab	NW2 Shower
99/11047	Biofilm swab	NW3 WC cistern
99/11048	Biofilm swab	NW3 Shower hose
99/11049	Net deposit	NW3 Kitchen cold tap
99/11050	Net deposit	NW2 Staff room cold tap
99/11051	10 litre	NW Hydrant 1
99/11052	10 litre	NW Hydrant 3
99/11053	10 litre	NW Hydrant 2
99/11054	Net deposit	NW Hydrant 1
99/11055	Net deposit	NW Hydrant 3
99/11056	Net deposit	NW Hydrant 2
99/11057	2.5 litre	RG1 Kitchen hot tap, closest to boiler
99/11058	2.5 litre	RG1 Bathroom hot tap
99/11059	10 litre	RG1 Shower water
99/11060	10 litre	RG1 Outside tap, closest to mains
99/11061	10 litre	RG1 Bathroom cold tap, from header tank
99/11062	Net deposit	RG1 Outside tap
99/11063	Biofilm swab	RG1 WC cistern
99/11064	Biofilm swab	RG1 Header tank
99/11065	Biofilm swab	RG1 Shower hose
99/11066	10 litre	RG Hydrant Horse close outside No. 9
99/11067	10 litre	RG Hydrant Queensway outside No. 39
99/11068	10 litre	RG Hydrant Briants Avenue outside No.17
99/11069	Net deposit	RG Hydrant Horse close outside No. 9
99/11070	Net deposit	RG Hydrant Queensway outside No. 39
99/11071	Net deposit	RG Hydrant Briants Avenue outside No.17
99/11072	2.5 litre	RG2 Kitchen hot tap, closest to boiler
99/11073	2.5 litre	RG2 Kitchen hot tap, furthest from boiler
99/11074	10 litre	RG2 Caretakers room cold tap, closest to mains
99/11075	10 litre	RG2 Classroom cold tap, furthest from mains
99/11076	Net deposit	RG2 Caretakers cold tap
99/11077	Biofilm swab	RG2 WC cistern
99/11078	Biofilm swab	RG2 Shower head
99/11079	2.5 litre	RG3 Kitchen hot tap, closest to boiler
99/11080	2.5 litre	RG3 Bath hot tap, furthest from boiler
99/11081	10 litre	RG3 Bathroom cold tap, furthest from mains
99/11082	10 litre	RG3 Kitchen cold tap, closest to mains
99/11083	Net deposit	RG3 Kitchen cold tap
99/11084	Biofilm swab	RG3 Shower head
99/11085	Biofilm swab	RG3 WC cistern
99/11086		NW water meter, job No. 84180411
99/11087		NW water meter, job No. 71910406
99/11088		NW water meter, job No. 96970418
99/11089		NW water meter, job No. 53420413
99/11090		NW water meter, job No. 48440420
WM 236	Sediment	Clarkes Hill Reservoir (bottom) 1
WM 237	Sediment	Clarkes Hill Reservoir (bottom) 2
WM 238	Sediment	Clarkes Hill Reservoir (bottom) 3
WM 239	Sediment	Clarkes Hill Reservoir (bottom) 4
WM 240	Swab	Clarkes Hill Reservoir (side) 1
WM 241	Swab	Clarkes Hill Reservoir (side) 2

Sample number	Sample type	Sample location
WM 243	10 litre	EL4 Mains supply classroom 2
WM 244	10 litre	EL4 Medical room cold tap from header tank
WM 245	2.5 litre	EL4 Hot tap nearest boiler
WM 246	2.5 litre	EL4 Hot tap furthest from boiler
WM 247	Net deposit	EL4
WM 248	Biofilm swab	EL4 WC cistern
WM 249	10 litre	Hydrant outside 15 Mulberry, E18
WM 250	10 litre	Hydrant outside 21 Laura close, E11
WM 251	10 litre	Hydrant outside 45 Arundel close, E11
WM 252	Net deposit	Hydrant outside 15 Mulberry, E18
WM 253	Net deposit	Hydrant outside 21 Laura close, E11
WM 254	Net deposit	Hydrant outside 45 Arundel close, E11
WM 255	10 litre	EL5 Cold tap kitchen, mains supply
WM 256	10 litre	EL5 Bathroom cold tap
WM 257	10 litre	EL5 Shower water
WM 258	2.5 litre	EL5 Kitchen hot tap, closest to boiler
WM 259	2.5 litre	EL5 Cleaners room hot tap furthest from boiler
WM 260	Net deposit	EL5 Mains tap
WM 261	Biofilm swab	EL5 Header tank
WM 262	Biofilm swab	EL5 WC cistern
WM 263	Biofilm swab	EL5 Shower water
WM 264	10 litre	EL6 Kitchen cold tap closest to mains
WM 265	10 litre	EL6 Bathroom furthest from mains
WM 266	10 litre	EL6 Shower water
WM 267	2.5 litre	EL6 Kitchen hot tap, closest to boiler
WM 268	2.5 litre	EL6 Bathroom hot tap, furthest from boiler
WM 269	Net deposit	EL6 Kitchen tap mains supply
WM 270	Biofilm swab	EL6 Shower head
WM 271	Biofilm swab	EL6 WC cistern
WM 328	10 litre	NW4 Bathroom mains cold tap
WM 329	10 litre	NW4 Kitchen mains cold tap
WM 330	10 litre	NW4 Header tank
WM 331	2.5 litre	NW4 Bathroom hot tap
WM 332	2.5 litre	NW4 Kitchen hot tap
WM 333	Biofilm swab	NW4 WC cistern
WM 334	Biofilm swab	NW4 Header tank
WM 335	Net deposit	NW4 Mains tap
WM 336	10 litre	NW5 Header tank
WM 337	10 litre	NW5 Shower water
WM 338	10 litre	NW5 Kitchen cold tap mains
WM 339	2.5 litre	NW5 Bath hot tap
WM 340	2.5 litre	NW5 Back bedroom hot water
WM 341	Biofilm swab	NW5 Shower head
WM 342	Biofilm swab	NW5 WC cistern
WM 344	Net deposit	NW5 Kitchen mains cold tap
WM 345	10 litre	NW6 Kitchen mains cold
WM 346	10 litre	NW6 Header tank
WM 347	2.5 litre	NW6 Bathroom sink hot tap
WM 348	2.5 litre	NW6 Kitchen hot tap
WM 349	Biofilm swab	NW6 Header tank
WM 350	Net deposit	NW6 Kitchen mains cold tap
WM 351	Net deposit	NW Hydrant 1 Thatchlead lane/Bury

Sample number	Sample type	Sample location
WM 352	Net deposit	NW Hydrant 2 Water street, Radcliffe
WM 353	Net deposit	NW Hydrant 3 jnct Heywood St/Kewshaw Rd
WM 354	10 litre	NW Hydrant 1 Thatchlead lane/Bury
WM 355	10 litre	NW Hydrant 2 Water street, Radcliffe
WM 356	10 litre	NW Hydrant 3 jnct Heywood St/Kewshaw Rd
WM 357	10 litre	RG4 Bath cold tap
WM 358	10 litre	RG4 Kitchen cold tap, mains supply
WM 359	2.5 litre	RG4 Kitchen hot tap
WM 360	2.5 litre	RG4 Bath hot tap
WM 361	10 litre	RG4 Shower water
WM 362	Biofilm swab	RG4 Shower head
WM 363	Biofilm swab	RG4 WC cistern
WM 364	Net deposit	RG4 Kitchen tap mains
WM 365	10 litre	RG Hydrant Briant avenue
WM 366	Net deposit	RG Hydrant Briant avenue
WM 367	10 litre	RG Hydrant Horse close
WM 368	Net deposit	RG Hydrant Horse close
WM 369	10 litre	RG Hydrant Queens way
WM 370	Net deposit	RG Hydrant Queens way
WM 371	10 litre	RG5 Header tank
WM 372	10 litre	RG5 Shower water
WM 373	10 litre	RG5 Cold tap, downstairs toilet/shower room, mains supply
WM 374	2.5 litre	RG5 Hot tap, bathroom sink
WM 375	2.5 litre	RG5 Hot tap, sink of downstairs toilet/shower room
WM 376	10 litre	RG6 Cold tap, caretakers office mains supply
WM 377	10 litre	RG6 Header tank water via classroom sink tap
WM 378	2.5 litre	RG6 Hot tap, caretakers office
WM 379	2.5 litre	RG6 Hot tap, staff toilet
WM 380	Net deposit	RG6 Cold tap, caretakers office
WM 381	Biofilm swab	RG6 WC cistern
WM 382	Net deposit	RG5 Mains tap
WM 383	Biofilm swab	RG5 Shower head
WM 384	Biofilm swab	RG5 WC cistern
WM 385	Biofilm swab	RG5 Header tank