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Deliverable D7.4

Report on lab-on-a-chip performance to detect DNA and RNA viruses

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1. Deviations from the original description of work

This deliverable is a part of work package 7 task 2, originally entitled "AquaTas: Development of a micro-Total Analytical System for on-site identification of contamination of water sources for human consumption" and later for reasons described below changed to "*Lab-on-a-chip*: Development of a micro*fluidic chip based assay* for on-site *detection of virus* contamination of water sources for human consumption (DTU-Food).

As already reported in the 1st and 2nd Periodic Report and Milestone 21, it has been necessary to make change to the original work description of this task. The chip system 'AquaTas', originally intended for use in this AQ WP7 task 2, was to be provided by experts in DELTA, who left DELTA and created the spin out company, Hygia Diagnostics ApS (HygiaDx), while still owing IP rights to the technologies covering the DELTA company's FlexChip technology. However, sufficient capital could not be raised in time by HygiaDx to continue development of this technology within the period of Aquavalens WP7. We were instead forced to use a back-up solution of an alternative chip, a microfluidic lab-on-a-chip based system (LOC) (COC Topas 5013L-10) with experts in our own institute, DTU-Food, and their collaboration with DTU-Nanotech and the Spanish company, POC. Thus we managed delivery of Milestone 21, demonstrating that it was possible to use the LOC system for detection of human adenovirus (HAdV) DNA in a solution of cell propagated HAdV and in fractions sampled during filter concentration of HAdV-spiked tap water.

However, during the continued work on the validation of HAdV chip detection and further chip detection of norovirus (NoV) and hepatitis A virus (HAV), we encountered new challenges as our contact with POC was interrupted following the close down of the company due to financial crisis in Spain in 2015. Therefore, we did not have either the chip/equipment or the intellectual property to continue the work with this chip. Due to this interruption in provision of chip/equipment and intellectual property, we were unable to further apply the chip for the validation of HAdV detection and for the development of the chip to include detection of the RNA viruses, NoV and HAV. Moreover, due to confidentially issues, it has also been extremely difficult to receive detailed information on the design and function of the chip system.

Thus, we have not fulfilled the complete aim of this deliverable D7.4, but are in the following providing a description of work that has been conducted in the attempt to reach the goal.

2. Introduction and revised aim

Enteric viruses, such as NoV causing gastroenteritis and HAV causing hepatitis are endemic in many areas of the world (Patel et al., 2009; Hall et al., 2011). They can infect the intestinal tract of humans via multiple environmental routes and are a major cause of waterborne diseases. People infected by NoV and HAV shed in very high numbers into the stools (Bosch 1998; Koopmans and Duizer 2004) and as the viruses have infectious doses, ranging from 1 to 100 viral particles (Koopmans and Duizer, 2004), water contaminated with very low concentrations of viruses represents a significant health risk. Therefore, sensitive methods to detect viruses in low concentrations in water samples are needed (Wu et al., 2011) to control and ensure the safety of water supplies.

As NoV and wildtype HAV are problematic to cultivate, detection relies on enzyme immunoassays or genome amplification (Hall et al., 2011). Presently, (RT-)PCR and (RT-)qPCR are the most commonly applied methods to monitor viral contamination in aquatic environment. Molecular tools are rapid, sensitive and specific, and they may provide valuable information for epidemiological studies via genotyping (Hamza et al., 2011). However, these methods are complex and expensive and mainly applicable in public health and food and environmental research laboratories, while of limited use in the field. Therefore, continued development of rapid, sensitive, and accurate point of analysis methods is required to allow simple and reliable detection of viruses in water.

Hence, to improve water safety, it would be of special interest for food and water management to enable analysis of NoV and HAV or suitable viral indicator for human wastewater pollution on-site where no laboratory facilities are available. An example of an often applied viral indicator is human HAdV. This is due to HAdV-40 and HAdV-41 being present in high amounts in faeces of young children with acute gastroenteritis (King 2012) as well as HAdVs being very persistent in water. Consequently, HAdV are considered to be a more conservative and suitable indicator of human viral fecal contamination (Jiang, 2006), than *e.g.* the traditional bacterial indicators, *E. coli* and intestinal enterococci.

The objective of this deliverable was to develop an on-site chip-based analysis system for virus detection and quantification and report on the performance of the chip to detect DNA and RNA viruses by describing the protocol(s) and performance evaluation of HAdV, NoV and HAV detection by microfluidic chip based assay. The LOC chip system which was under development for detection of bacterial pathogens on meat samples at point-of-analysis (PoA) should first be adapted to detect and quantify DNA viruses (HAdV) and then further be developed for the detection of the important food and waterborne RNA viruses (NoV and HAV). The efficiency should be evaluated against regular Taqman (RT-)qPCR with focus on sensitivity. In case of promising results the detection system should be assessed for the ability to detect and quantify these viruses in extracts (from WP6) of different water types with focus on sensitivity and inhibition.

3. Material and methods

3.1. Lab-on-a-chip system for detection of virus

This alternative LOC, fabricated at DTU Nanotech (DTU, Department of Micro and Nanotechnology), consist of a 1x1x0.1 cm prototype polymeric chip with an injection molding insert of hard aluminum (alloy 2017, MetalCentret, Denmark) using a micro-milling system (Folken Ind., Glendale, California, USA). The chip with a 12 µL chamber volume was replicated in the high refractive index thermal plastics i.e., COC Topas 5013L-10 using an injection molding system Victory 80/45 Tech (Engel, PA, USA). On-chip PCR detection of virus was carried out using a flatbed thermocycler with the primers described below. The size of amplified products were visualized and confirmed on ethidium bromide stained agarose gels under UV light.

The system was originally developed for detection of Avian Influenza virus and bacterial pathogens, but was in present project altered to allow detection of HAdV and tested for its feasibility to detect HAdV in extracts of spiked tap water.

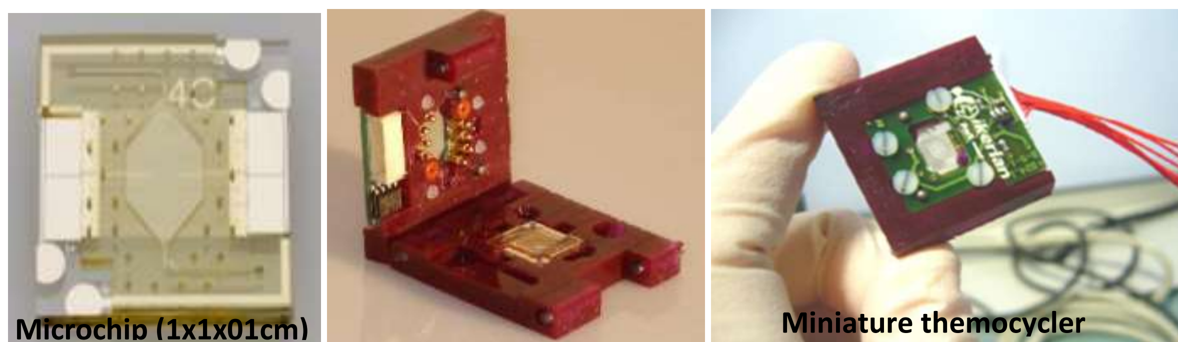

Figure 1. Pictures of the polymeric chip with dimension 1x1x0.1 cm and miniature thermocycler.

3.2. Preparation of viral reference material

For the development and performance testing of the chip's ability to detect viruses, different types of DNA (for HAdV) and RNA (for NoV and HAV) reference material were prepared and quantified by (RT-)qPCR defined as genome copies (GC) per µl of reference material.

3.2.1. DNA virus, HAdV

For detection of DNA viruses, the following HAdV reference material was prepared and quantified:

1. HAdV-41 dsDNA artificially constructed segments (273 bp) (gBlocks® Gene Fragments, Integrated DNA Technologies)
2. HAdV-35 DNA extracted from whole particles propagated in culture of 293A cells by following the instruction manual of the supplier (Invitrogen, Life technologies)
3. Nucleic acid extracts of virus concentrated from 10 L samples of tap water spiked with HadV-35.

To select the most efficient qPCR assay for quantification of both HAdV-35 and HAdV-41 in reaction volumes suitable for the chip (preferably 12 µl), we compared two traditional qPCR assays developed by Hernroth *et al., (*2002) and Jothikumar *et al.,* (2005).

Detection of HAdV was performed on HAdV-41 dsDNA constructs and HAdV-35 genomic DNA extracts from cell-cultured HAdV-35 using the NucliSens reagents and miniMag system (Biomerieux). qPCR reactions were performed in duplicate reaction on a 96-well plate format of ABI Step One (Applied Biosystems) using the TaqMan® Environmental Universal PCR Master Mix (ABI, Life Technologies). Amplification was carried out in a total of 10, 15 and 25-µl reaction mixtures containing 2.5 µl DNA. Applied primers and probe and concentrations of these were as described by Hernroth et al. (2002), 0.9µM of each primer Ad-F and Ad-R, and 0.225 µM of probe Adp1, or by Jothikumar *et al.* (2005), 0.25 µM of each primer JTVXF and JTVXR, and 0.15 µM of probe JTVXP. In both assays the applied reaction conditions were as described by Hernroth, 95°C for 10 min, 45 cycles of 95°C for 15 sec and 60°C for 60 sec. qPCR quantification and determination of lowest possible detection (LOD) of HAdV-35 virus stock was performed by interpolating Ct values to standard curves generated from end-point detection of 10-fold dilution series ranging from 7.38×10^0 – 7.38×10^5 GC/reaction measured by cubit of HAdV-41 dsDNA segments.

To test the chip's ability to detect HAdV extracted from tap water, reference material for this was prepared using concentration and extraction methods developed in WP6. Here, HAdV-35 (10^6 GC)

were spiked in two portions of 10 L of tap water and concentrated from each portion by either the glass wool filtration or the monolithic affinity filtration method (see D6.3 for method details), and nucleic acids were extracted using the NucliSens/miniMag extraction system. The recovered HAdV-35 was quantified as described above, but in 25 µl reaction volumes using the HAdV qPCR assay developed by Hernroth *et al.* (2002).

3.2.2. RNA viruses, NoV and HAV

For NoV genogroup (G)I and GII and HAV, the following reference material was prepared and quantified:

1. Transcript RNA (3 kb) of NoV GI.3b and GII.1 (Gentry *et al*., 2009).
2. Extracted viral RNA of NoV GI.1, GI.8, GII.3 and GII.4 obtained from positive stool samples collected during outbreak investigations and of HAV (ATCC HM175/18f) propagated in FRhK-4 cells (De Medici et al., 2001).
3. NoV GI (125 bp), NoV GII (125 bp) and HAV (216 bp) artificially constructed dsDNA (gBlocks® Gene Fragments).

NoV and HAV were quantified by RT-qPCR according to the European standard method for virus detection in food (ISO/TS 15216-1). In brief, when appropriate, viral RNA was extracted as described above for HAdV DNA (section 3.2.1). NoV and HAV genomes were quantified in duplicate reactions on an ABI Step One using the RNA UltraSense one-step qRT-PCR system (Invitrogen) according to the manufactures instructions and by using 5 µl RNA/DNA and the following reaction conditions, 55 °C for 1 min, 95 °C for 5 min and 45 cycles of 95 °C for 15 s, 60 °C for 1 min, and 65 °C for 1 min. The applied forward and reverse primers and probes, were for NoV GI, QNIF4, NV1LCR, NVGG1p, for NoV GII, QNIF2, COG2R, QNIFS, and for HAV, HAV68, HAV240 and HAV150 (ISO/TS 15216-1). As standard curves 5 µl of 10-fold dilution series of transcript RNA of NoV GI.3b (ranging 2×10^0 - 2×10^3 GC/reaction), GII.1 (ranging 3×10^0 - 3×10^3 GC/reaction) or viral RNA of HAV (ranging from 0.5×10^0 - 5×10^2 RT-PCR Units/reaction) measured by cubit were used for quantification.

3.3. Detection of HAdV by lab-on-a-chip

To test the LOC system for the ability to detect HAdV, a number of experiments were conducted, using the primers and PCR conditions as applied for the qPCR assay developed by Hernroth et al., (2002), described in section 3.2.1., except that the total PCR reaction volume (incl. death volume) was 12 µl. The following experiments were conducted:

a) Inhibition from the polymer chip itself during amplification was tested using triplicate 7.5×10^5 GC of artificially constructed 273 bp dsDNA HAdV-41.
b) Determination of the lowest number of HAdV-41 dsDNA that could be detected on the chip system was tested on 10 fold series dilution (ranging from 7.5×10^5 - 7.5×10^{-1} GC/reaction) of HAdV-41 dsDNA constructs.
c) The lowest number that could be detected of HAdV-35 was tested on 10-fold dilution series (ranging from 7.3×10^4 - $7.3\times10\text{-}^1$ GC/reaction) of DNA extracts derived from cell propagated HAdV-35. In the same experiment we tested the ability of the chip to detect DNA extracts of HAdV-35 that was concentrated by Monolithic affinity filtration from 10 L tap water spiked with 10^6 GC HAdV-35.
d) The ability to detect HAdV DNA in extracts from sample fractions (filtrate, eluate and concentrate) taken during glass wool filtration of HAdV-35 (10^6 GC) spiked 10 L of tap water was

tested. In this experiment a repeated test of the chip to detect DNA extracts of HAdV-35 concentrated by Monolithic affinity filtration from 10 L tap water spiked with 10^6 GC HAdV-35 were conducted.

4. Results

4.1. Detection of HAdV in reference material by qPCR

The two RT-qPCR assays described by Hernroth et al (2002) and Jothikumar et al (2005) were evaluated for detection and standard curve performance of HAdV-41 dsDNA constructs. While the Jothikumar assay, opposed to the Hernroth assay, in three occasions failed to provide useful standard curves for HAdV-41 dsDNA constructs (data not shown), the Hernroth assay was selected for further evaluation on performance in different volumes of reaction mix. Results of standard curve formula and limit of quantification (LOQ) for HAdV-41 dsDNA constructs and lowest LOD for HAdV-35 extracted DNA of viral propagated cell culture using reaction mixtures of 10, 15 or 25 µl are shown in Table 1. As can be seen, the slope, intercept, R2 and LOQ values for qPCR detection of HAdV-41 dsDNA constructs are comparable regardless of the applied reaction volume, and so is the determined concentration and LOD of cell cultured HAdV-35 DNA extracts. As the Hernroth assay was found to provide linear and efficient amplification of both HAdV-41 and HAdV-35 end-point detection, this assay was chosen for further use.

Thus using this assay to determine recovered HAdV-35 in filtrate, eluate and concentrate after glass wool filtration of HAdV-35 spiked 10 L tap-water, resulted in Ct values of 40.75, 37.82 and 33.45, corresponding to 0.3, 2.0 or 37 HAdV-35 GC/reaction, respectively, which can be calculated to 1×10^5, 2×10^4 or 2×10^3 recovered HAdV-35 GC/sample fraction, respectively.

Table 1. Results obtained by qPCR (Hernroth et al., 2005) determination of HAdV reference material prepared for on-site chip detection.

qPCR reaction volume (µl)	HAdV-41 sdDNA		HAdV-35 vDNA	
	Standard curve formular – Slope, intercept and R2	LOQ (GC/reaction)	Stock concentration	LOD (GC/reaction)
10	$Y = -3.5566x + 39.196$; $R^2 = 0.9949$	7.4	$2.6\times10^6 \pm 7.2\times10^5$	8.2
15	$Y = -3.4572x + 38.885$; $R^2 = 0.9993$	7.4	$2.9\times10^6 \pm 6.9\times10^5$	11.6
25	$Y = -3.5181x + 39.900$; $R^2 = 0.9975$	7.4	$3.2\times10^6 \pm 1.3\times10^6$	<14.2

4.2. Detection of NoV and HAV in reference material by qPCR

RT-qPCR detection of transcript RNA for NoV, viral RNA for HAV and artificially constructed dsDNA for NoV and HAV from series of 10-fold dilutions performed as shown in table 2. Based on the standard curves for the NoV transcript RNA, RNA extracted from series 10-fold diluted stool samples positive for NoV allowed efficient amplification of as low as 5.99 ± 1.62 GII.3 GC/reaction, 2.50 ± 0.96 GII.4 GC/reaction, 9.21 ± 0.78 GC GI.1/reaction and 4.98 ± 4.10 GC GI.8 /reaction, thus these detection limits and the LOQ-values from the table was to be compared by the detection limit that could be obtained on the same respective viral reference material using the LOC.

Table 2. Results obtained by qPCR (ISO/TS 15216-1) determination of NoV and HAV reference material prepared for on-site chip detection.

Virus	Standard curve formula: Slope, intercept and R^2	LOQ (GC/reaction)
NoV GI.3b tRNA	Y = -3.7453x + 41.079; R^2 = 0.9938	2.2
NoV GII.1 tRNA	Y = -3.3028x + 37.175; R^2 = 0.9924	2.9
HAV vRNA	Y = -3.3758x + 34.760; R^2 = 0.9995	21
NoV GI dsDNA	Y = -3.3900x + 38.278; R^2 = 0.9948	22
NoV GII dsDNA	Y = -3.5570x + 38.372; R^2 = 0.9920	2.8
HAV dsDNA	Y = -3.4656x + 40.353; R^2 = 0.9917	0.41

4.3. On-chip detection of HAdV in reference material by lab-on-a-chip

The performance of on-chip PCR detection of HAdV was tested in a conventional PCR format on the LOC using flatbad thermocycler and 12-µl reaction volumes with the primers and reaction conditions described in the Hernroth qPCR assay (Hernroth *et al.,* 2002). Amplicons were run on agarose gels to visualize detection. Overall, this resulted in similar or even better detection efficiencies as obtained with the qPCR; as approximately 1 GC/reaction of both HAdV-41 DNA constructs and HAdV-35 DNA extracted from whole cell cultured viruses could be detected with no signs of inhibiting effect during amplification as shown in Figure 2-4.

Thus no PCR inhibition was observed when testing the inhibiting effect of the polymer chip itself during triplicate amplification of 7.5×10^5 GC of artificially constructed 273 bp dsDNA HAdV-41 (Figure 2). When testing 10 fold series dilution (7.5×10^5 - 7.5×10^{-1} GC per reaction) of HAdV-41 dsDNA constructs, HAdV could be detected in all dilutions indicating a LOD below 1 GC/reaction (Figure 3). Likewise all 10-fold dilutions tested (7.3×10^4 - 7.3×10^{-1} GC per reaction) of HAdV-35 extracts from cell culture could be amplified, which also corresponds to a LOD lower than 1 GC/reaction (Figure 4).

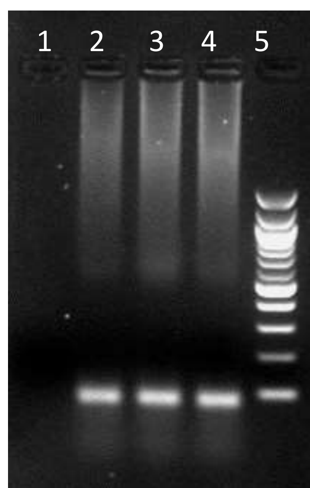

Figure 2. *Testing for inhibition by the polymer chip. Lane 1: Negative control; Lane 2-4: PCR amplified products from HAdV-41 dsDNA constructs; Lane 5: 100 bp ladder molecular weight marker (2µl).*

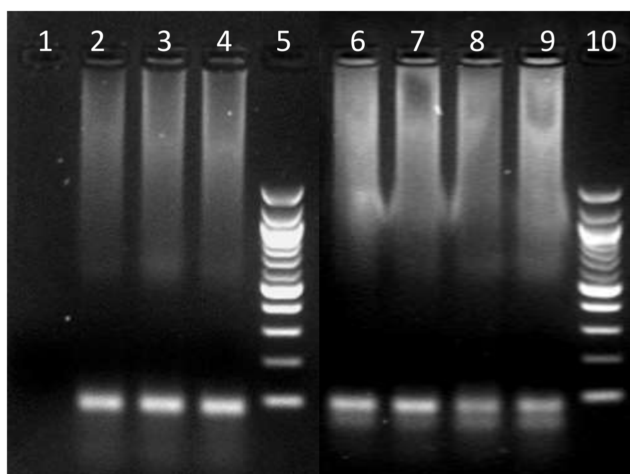

Figure 3. On-chip PCR amplification of HAdV-41 dsDNA constructs in series 10-fold dilutions. Lane 1, Negative control; lane 2, 7.5×10^{5}*; Lane 3,* 7.5×10^{4}*; Lane 4,* 7.5×10^{3}*; Lane 5, 100 bp lader; Lane 6,* 7.5×10^{2}*; Lane 7,* 7.5×10^{1}*; Lane 8,* 7.5×10^{0}*; Lane 9,* 7.5×10^{-1}*; Lane 10, 100 bp ladder.*

Moreover the chip system allowed detection of HAdV DNA in extracts from 10 L tap water spiked with HAdV-35 (10^{6} GC) after concentration by monolithic affinity filtration developed in task 6.2 (Figure 4 and 5). During the development of the method for glass wool concentration of viruses in WP6, varying degree of inhibition was observed during HAdV-35 qPCR detection in extracts from glass wool filtrates, eluates and concentrates of 10-L HAdV-35 (10^{6} GC) spiked tap water (see Ct-values and corresponding detected concentrations of GC's in section 4.2). To get an indication whether the inhibiting components in the extracts affected the on-chip detection as well, the same extracts were chosen to be tested in the on-chip PCR amplification format. Although week bands, the chip system resulted in HAdV detection in extracts of filtrate, eluate as well as concentrate obtained by the glass wool filtration (Figure 5).

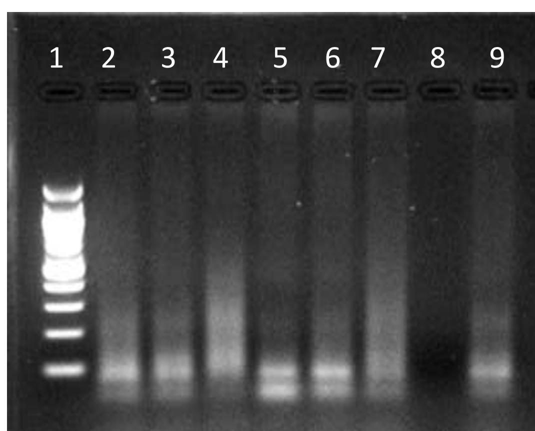

Figure 4. On-chip PCR amplification of HAdV-35 DNA from viral extracts of cell culture in series 10-fold dilutions and in extract of HAdV-35 spiked 10 L tap water concentrated by monolithic affinity filtration. Lane 1, 100 bp ladder; lane 2, 7.3×10^{4}*; Lane 3,* 7.3×10^{3}*; Lane 4,* 7.3×10^{2}*; Lane 5,* 7.3×10^{1}*; Lane 6,* 7.3×10^{0}*; Lane 7,* 7.3×10^{-1}*; Lane 8, Negative control; Lane 9, Extract of 10 L tap water spiked with HAdV-35 (*10^{6} *GC) and processed using monolithic affinity filtration.*

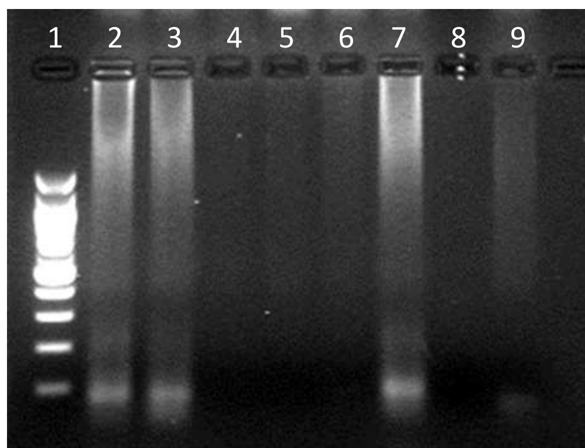

Figure 5. On-chip detection of HAdV-35 DNA extracts of spiked tap water concentrated using glass wool filtration and monolithic affinity filtration. Lane 1, 100 bp ladder; lane 2, Filtrate of glass wool filtration; Lane 3, Eluate of glass wool filtration; Lane 4, 5 , 6: Leak in chambers resulting in lost PCR products; Lane 7, concentrate of glass wool filtration; Lane 8, Negative control; Lane 9, Concentrate of monolithic affinity filtration.

Thus, the detection of HAdV DNA using the LOC was shown to have approximately the same detection efficiency as the use of traditional qPCR conditions and thermocycler whether it originates from artificially constructed dsDNA segments of HAdV-41 or extracts of whole HAdV-35 virus from cell culture and from fractions of water concentrates.

4.4. On-chip detection of NoV and HAV in reference material by LOC

As explained in section 1, we were unable to complete this part of the aim due to interruption in provision of chip/equipment.

5. Discussion

With this work we have adapted the LOC system to detect and quantify DNA viruses (HAdV) and evaluated the efficiency of performance against Taqman (RT-)qPCR with focus on sensitivity and inhibition. In addition, we also assessed the LOC system for the ability to detect HAdV in extracts (from WP6) of different water fractions during concentration. However, despite that all the reference material was prepared, we did not manage to further develop the system to detect the RNA viruses (NoV and HAV).

Although the results of the experiments would have benefitted by being repeated, we have with this study shown that it is possible to use the LOC system for detection of HAdV DNA in solutions of artificially constructed dsDNA as well as in extracts of HAdV from cell culture and in fractions sampled during filter concentration of HAdV-spiked tap water following nucleic acid extraction using the NucliSens/miniMag system. For the HAdV detection of dsDNA constructs and of viral concentrates from cell cultures the sensitivity of detection was comparable to or even better than the broadly applied qPCR assay for HAdV (Hernroth *et al.,* 2002).

The whole diagnostic LOC procedure and PCR amplification were accomplished in a single chamber and the system was capable to detect both HAdV-41 and HAdV-35 at approximate concentration of 1 GC/reaction within 40 min. The simple design of the chip, together with high level of integration,

thermocycling PCR amplification in short time (26 min shorter than the corresponding qPCR), is a valued practical applicability of the LOC system. Therefore it may facilitate rapid on-site screening of HAdV as viral indicator for waste water pollution for applications in food and water safety control. As HAdV can also cause infections in vulnerable people and children less than 10 years (King 2012), detection of HAdV would in addition to serve as a viral indicator of sewage pollution also indicate potential presence of pathogens.

In order for the LOC system to be applicable to test for other pathogenic viruses such as the RNA viruses, NoV and HAV, it would need an RT step prior to the PCR, which should be fairly easy to accomplish on the flatbed thermocycler. However, as RT reaction is more vulnerable to inhibition than PCR reaction, it is likely that RT-PCR detection of target RNA viral genomes in water extracts will result in some level of inhibited detection performance. Unfortunately we were not able to address this part of the goal for reasons described in section 1.

6. Conclusion

We have applied a prototype microfluidic lab-on-chip system for detection of HAdV and showed that a performance comparable with traditional pPCR could be achieved. Unfortunately, we were unable to complete the evaluation of the chip detection of HAdV and the last part of the deliverable, the chip detection of RNA viruses, due to the sequential series of challenges in the delivery of chip/equipment and maintenance of intellectual knowledge.

7. References

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8. Implications of the results of Deliverable 7.4

Implications of the results of Deliverable Report 7.4

Implications of the results for the Work Package (WP 7)

In WP7 a variety of different detection methods were developed, without dependencies for each other, and only a few of them were chosen for the field studies e.g. task 7.1 “Standardised molecular detection”. The LOC detection of HAdV reported here was a complement to task 7.1. With this deliverable we used a prototype microfluidic lab-on-chip for detection of HAdV. Most importantly, the use of the LOC system showed that it may facilitate development of an alternative detection system of HAdV without loss in sensitivity compared to qPCR. In addition, the smaller reaction volume and the shorter PCR amplification time necessary for the LOC system may reduce time and hand on work compared to detection by other PCR-methods. However, we have not been able to explore and demonstrate the applicability of the chip system to detect RNA viruses such as NoV and HAV.

Implications of the results for this Cluster 2

This task did not have any dependencies within the cluster. However, as detection of HAdV could be achieved with this LOC system, it may also be applicable for adaptation to the other PCR assays developed and evaluated in cluster 2.

Implications of the results for the whole project

As cluster 3 in their field studies has chosen alternative detection methods (qPCR) combined with methods for water concentration (WP6), the results obtained in this task will not have any implications for cluster 3.

Indicate key external stakeholders interested in the results of Deliverable Report 7.4

Rapid chip based detection for a HAdV could be of interest for water managers. However, before it is useful for stakeholders, the system will need continued development before completion as well as for the detection of RNA viruses.

Which internal partners should your deliverable be sent to?

Cluster 2 leads and partners working on microfluidic chip detection (University of Herriot Watt)