



**Deliverable D-JRP12-WP 3.2, 3.3,
3.4 & 3.5**

Report on initial findings and protocols for on-site diagnostic tests and IT solutions for human clinical, veterinary and environmental samples for early warning of emerging resistant pathogens.

JRP12-AMRSH5-FARMED

Contributing Partners:

APHA, DTU, IZSAM, Sciensano, WBVR



GENERAL INFORMATION

European Joint Programme full title	Promoting One Health in Europe through joint actions on foodborne zoonoses, antimicrobial resistance and emerging microbiological hazards
European Joint Programme acronym	One Health EJP
Funding	This project has received funding from the European Union's Horizon 2020 research and innovation programme under Grant Agreement No 773830.
Grant Agreement	Grant agreement n° 773830
Start Date	01/01/2018
Duration	60 Months

DOCUMENT MANAGEMENT

Project deliverable	D-JRP12-WP 3.2, D-JRP12-WP 3.3, D-JRP12-WP 3.4, D-JRP12-WP 3.5
Project Acronym	JRP12-AMRSH5-FARMED
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Other contributors	
Due month of the report	M60
Actual submission month	M60
Type <i>R: Document, report DEC: Websites, patent filings, videos, etc.; OTHER</i>	R, DEC, other Save date: 30-Dec-22
Dissemination level <i>PU: Public (default) CO: confidential, only for members of the consortium (including the Commission Services)</i>	PU This is the default setting. If this project deliverable should be confidential, please add justification here (may be assessed by PMT): Work from this deliverable is being prepared for publication.
Dissemination <i>Author's suggestion to inform the following possible interested parties.</i>	<p>OHEJP WP 1 <input type="checkbox"/> OHEJP WP 2 <input type="checkbox"/> OHEJP WP 3 <input checked="" type="checkbox"/></p> <p>OHEJP WP 4 <input checked="" type="checkbox"/> OHEJP WP 5 <input type="checkbox"/> OHEJP WP 6 <input type="checkbox"/></p> <p>OHEJP WP 7 <input type="checkbox"/> Project Management Team <input type="checkbox"/></p> <p>Communication Team <input checked="" type="checkbox"/> Scientific Steering Board <input checked="" type="checkbox"/></p> <p>National Stakeholders/Program Owners Committee <input type="checkbox"/></p> <p>EFSA <input type="checkbox"/> ECDC <input type="checkbox"/> EEA <input type="checkbox"/> EMA <input type="checkbox"/></p> <p>FAO <input type="checkbox"/> WHO-Europe <input type="checkbox"/> OIE <input type="checkbox"/></p> <p>Other international stakeholder(s):</p> <p>Social Media:</p> <p>Other recipient(s):</p>



1. Introduction

Antimicrobial resistance (AMR) is one of the greatest global public health concerns in modern times. Development of tools for real-time detection of antimicrobial resistant (AMR) pathogens is a priority topic of the One Health EJP. Such tools will provide clinicians and veterinarians with appropriate information aiding in applying effective treatments and management practices. For real-time analysis to be achievable on-site (away from the laboratory setting), robust culture independent detection methods, employing minimal equipment are required. Metagenomic sequencing techniques can detect the composition of microbial communities for the assessment of potential pathogens and AMR or virulence genes, which could become an invaluable diagnostic tool. Currently, there are two sequencing technologies, short and long read methodologies. Unlike the current short read technologies that are dependent on large laboratory instruments, Oxford Nanopore Technology (ONT) offers a portable long read sequencing technology, for on-site metagenomics to identify the bacterial community ('microbiome') and associating the genetic context of target genes such as those conferring antimicrobial resistance ('resistome').

The FARMED project aimed to assess the feasibility of this long read sequencing technology, as offered by Oxford Nanopore Technology (ONT). In addition to feasibility, FARMED aimed to explore the applicability of long read metagenomics to be conducted on-site. A literature review defining four levels of on-site DNA sequencing as well as current commercially available methods was conducted in 2020 (deliverable D-JRP16-WP3.1 <https://zenodo.org/record/4724557>). This WP3 deliverable summarizes the initial findings on mock and on-site diagnostic tests that were performed to analyse human clinical, veterinary, and environmental samples to detect the microbiome and resistome. Long read sequencing is dependent on the ability to perform on-site DNA extraction generating DNA sufficient quantity and quality to perform ONT MinION sequencing. Protocols used in this report are based on the findings described on the initial feasibility studies performed as part of WP1 (<https://zenodo.org/record/7429361>). Furthermore, IT solutions are based primarily on the outcomes for on-site sequencing as described in WP2 (<https://zenodo.org/record/7433258>). WP3 includes the outcomes and appendices for the mock on-site and on-site protocols used for:

- DNA extraction methods using adapted or on-site commercially available system,
- Library preparations, using the VolTrax V2 system, an ONT solution for on-site library preparation,
- ONT long read sequencing protocols.

2. Tests performed for on-site DNA extraction methods

The input DNA is an essential part of the on-site analysis, which has been proven more challenging than initially predicted, as described in the literature review on this topic <https://doi.org/10.5281/zenodo.4724557>. Several methods have been tested since, as described below.

2.1. *Claremont Biosolutions DNAexpress™ kit*

At In-6, development of an on-site DNA extraction protocol started from the Claremont Biosolutions DNAexpress™ kit (<https://www.claremontbio.com>), which uses a battery-powered, microtube-sized bead-beating device to homogenize the sample and lyse cells. The protocol was modified after several iterations, until an on-site protocol was obtained that consistently resulted in DNA extracts of high molecular weight, yield, and purity. To assess DNA purity, the 260/280 nm and 260/230 nm absorbance ratios were measured by Nanodrop (ThermoFisher Scientific). DNA fragment lengths were determined by the TapeStation genomic DNA assay (Agilent). DNA concentrations were determined using the Qubit



dsDNA HS assay (ThermoFisher Scientific). The results of the different versions of the protocol are summarized in table 1.

First, the DNAexpress protocol was performed as per manufacturer instructions, which resulted in high yields for pure bacteria and pure bacterial mock communities, although fragment sizes were low in comparison with other extraction methods. Additionally, the DNA purity proved highly variable between different extractions. For these reasons, an extra DNA purification step using AMPure XP beads (Beckman Coulter) was included. While purity improved for pure bacteria, it did not for faecal DNA extracts. DNA integrity improved in both cases; this was likely due to the AMPure XP bead purification step and the size-selection effect.

To further improve the protocol, several other modifications were made, for example using a reduced-voltage battery to power the bead-beating step and replacing the filter-column based DNA purification steps with the purification steps of the Zymo QuickDNA HMW magbead protocol. Finally, this resulted in an on-site applicable protocol that consistently provided high quality, high molecular weight DNA extracts (appendix 2). The method requires a vortex, hula sample mixer, magnet, centrifugation, and heating at some steps of the protocol. The latter two can be done on-site with the Bento Bio Pro portable laboratory device (<https://bento.bio/devices/>), which includes a microcentrifuge and thermocycler block.

Table 1. Iterations of the on-site protocol using DNAexpress

Sample	Extraction protocol	A260/280	A260/230	Fragment length (bp)	Extracted DNA (ng)
<i>E. coli</i> culture (10 mL overnight culture)	DNAexpress according to manufacturer's instructions	1.81	1.4	18000	5120
75 µL Zymo GMS	DNAexpress + AMPure XP bead purification	2.03	1.68	14400	658
100 mg chicken faeces	DNAexpress + AMPure XP bead purification	1.69	1.00	11200	232
75 µL Zymo GMS spiked in 100 mg chicken faeces	Modified DNAexpress protocol + AMPure XP bead purification	1.81	1.28	9800	1530
100 mg chicken faeces	On-site protocol: Modified DNAexpress + QuickDNA HMW magbead + AMPure XP bead purification	1.89	2.37	27300	590
75 µL Zymo GMS spiked in 100 mg chicken faeces	On-site protocol performed using the Bento Bio Pro: Modified DNAexpress + QuickDNA HMW magbead + AMPure XP bead purification	1.88	1.88	38100	1785

2.2. *BentoLab & Quick-DNA HMW MagBead*

Institute 2 tested the on-site DNA extraction from faecal and soil samples (metagenomics). Extractions were done using Quick-DNA HMW MagBead Kit (D6060, Zymo Research) on BentoLab device for DNA extraction and library preparation (<https://bento.bio/devices/>) with an external portable power supply. The extraction was done following the manufacturer's instructions with the following modifications.

All incubations were done in PCR tubes (BentoLab has a single heat block for PCR tubes only), and all centrifugations were done at 5000 x g with increased centrifugation time on all steps (doubled). All bead cleaning and elution steps were done using Invitrogen MagRack. As a control, *Staphylococcus*



aureus bacterium was added to the faecal sample (100 µl of 10⁸ cells/mL). DNA concentrations were 23.6 ng/L and 19.4 ng/L for faeces and soil respectively.

2.3. *E.Z.N.A.® Stool DNA Kit (Omega Bio-tek)*

Institute 3 selected poultry farms for the on-site experiment. The aim was to combine the standard sample collection protocol (bootsocks), generally coupled with culture methods for *Campylobacter* and *Salmonella*, with a metagenomic approach for the detection of pathogens, and AMR genes along with microbiota characterization from faeces/environment. Controls were prepared in a pre-starting session collecting poultry meconium (one-day old chickens) using bootsocks. The absence of *Campylobacter* and *Salmonella* was proven by culture methods (ISO 10272/2017 and ISO 6579/2017), thus positive control was made spiking with 10⁸ cells of both pathogens into the “negative” bootsocks. The same protocol was used on-site for the bootsocks collected in 6 different poultry flocks after 45 days housing. The E.Z.N.A.® Stool DNA Kit from Omega Bio-tek was selected for on-site DNA extractions from the bootsock samples, which generated DNA concentration ranging from 3 to 10.6 ng/µl (50 µl), DNA Integrity (DIN) >6 (6 – 6.7) and average size of 26 Kb (24.7 – 27.8 Kb) (Table 2). The DNA extraction method selected for on-site experiments gave good results in terms of integrity and fragment size, while DNA concentrations were low.

Table 2. Details of the DNA samples extracted by the E.Z.N.A.® Stool DNA Kit (Omega Bio-tek)

ID sample	Qubit ng/µl	Tapestation peak Size	Tapestation DIN
BS01	10.6	24704	6.4
BS02	3.7	25057	6
BS03	3	27210	6.4
BS04	4.9	27792	6.7
BS05	4.1	25446	6.6
BS06	8.8	25420	6.5

2.4. *PDQeX DNA extraction platform (MicoGEM, UK)*

The PDQeX DNA extraction platform (MicoGEM, UK) was used by two institutes, to extract DNA from milk and faecal samples from different livestock. The PDQeX is a system for automated DNA extraction (figure 1), which utilises a single use extractor cartridge. The cartridge uses temperature to activate a mixture of thermophilic enzymes that lyse cells and destroys nucleases and proteins, leaving nucleic acid, which can be used for several molecular biology techniques such as PCR and whole genome sequencing. The PDQeX platform is considered a convenient option for on-site DNA isolation due to the minimal need for other equipment and limited hands-on time.

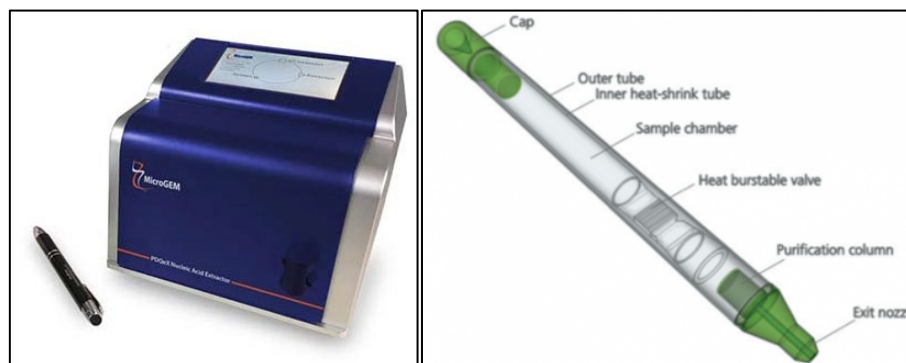


Figure 1. Left panel: Microgem PDQeX portable and automated DNA extractor. Right Panel: PDQeX uses a closed tube system which handles all steps of DNA extraction.



2.4.1. DNA extraction from milk

Metagenomic sequencing of milk could provide a quick alternative in mastitis diagnostics. Raw cow milk, spiked with mastitis pathogens was therefore tested for metagenomic extraction with the PDQeX method, using a range of volumes, see table 3. Tests will be further conducted for on-site DNA extraction using 1 ml input material due to the convenience of the smaller centrifuge needed.

Table 3. Extraction of metagenomic DNA from raw cow milk, n = 3 spiked raw milk samples.

	0.25 ml	0.5 ml	1 ml	2 ml	5 ml
Average	0.11 ng/μl	0.26 ng/μl	0.65 ng/μl	0.89 ng/μl	0.61 ng/μl
Range	0.07-0.14	0.16-0.37	0.38-0.83	0.69-1.10	0.42-0.69

2.4.2. DNA extraction from livestock faeces

Initial trials of the PDQeX system at In-1, using the manufacturer’s protocol, to extract DNA from faeces spiked with *E. coli* and MRSA, required further optimisation to increase DNA yields. In partnership with the manufacturer, a new prototype of the extraction tubes, increasing the sample input amounts (20mg to 100mg) and extraction conditions, appear to have increased DNA yield as well as recovering long DNA fragments (>12kb), although still not optimal (~10 ng/μl) for MiniON sequencing which requires ~50ng/μl.

Following several iterations of faecal DNA extraction, a methodology has been developed that gave consistent DNA concentrations, which included heating the samples to 40 °C for 10 minutes prior to commencing the DNA extraction. However, as the concentration for one extraction does not yield enough DNA for the rapid barcoding kit, the sample has to be extracted four times and pooled. It was also found that the eluted DNA from PDQeX extractions was too 'dirty' containing proteins which during nanopore sequencing would rapidly destroy the sequencing pores and prematurely end sequencing of the DNA samples. Therefore, a clean-up method was derived utilising two types of magnetic beads in a 1:1 ratio with the volume of eluted DNA following the precipitation of DNA to maximise the recovered yield. The DNA analyser at WBVR, Tapestation, showed that using a combination of two different beads as well as use of absolute ethanol increased the yield of DNA fragments over 5000 bases. Following magnetic bead clean up, DNA was suitably clean to be processed through a flow cell and produce DNA sequences that could be used to determine the bacterial species and AMR genes within the faecal samples. DNA samples were sequenced, using the rapid barcoding kit, singleplex per flow cells, for approximately 13 hours, and allowed several samples to be run sequentially on the same flow cell (assuming enough pores on the flow cells could be recovered). The method devised at In-1 was not performed on-site but was mostly followed during the on-site sequencing test performed by In-8.

At In-8, DNA extraction from two faecal samples per animal species were conducted using the PDQeX (tested in duplex) and compared to the Purelink Microbiome DNA isolation kit, which is the current standard for DNA extraction from faeces (Table 4). Although the Purelink extraction currently results in a higher yield and more reproducible results between samples, the PDQeX extracted DNA was of sufficient quality and quantity for downstream sequencing.

Table 4. Extraction of metagenomic DNA from livestock faeces.

	PDQeX average n=4 (range)	Purelink average n=2 (range)
Pig	3.2 ng/μl (2.4-3.8)	12.1 ng/μl (11.7-12.5)
Veal	2.6 ng/μl (1.4-4.7)	10.9 ng/μl (10.9-11.0)
Broiler	7.3 ng/μl (4.5-10.8)	11.9 ng/μl (11.6-12.2)

Although we had mixed success, the PDQeX system does offer a safe method for extraction of DNA



from milk and faecal material, however the resulting DNA requires further purification to ensure it is not detrimental to the flow-cell. Currently, the method still requires optimisation to increase the concentration of DNA as well as remove impurities which prematurely destroy the flow cells during sequencing. The method can be performed with minimal laboratory equipment requiring access to a power source. In addition to DNA quantity, careful consideration must be given to quality of DNA used in MinION sequencing, which may not be a critical for other molecular biology-based methods such as PCR methods.

3. Tests performed using the VolTrax V2 system

At the onset of the FARMED project, the VolTrax V2 system was expected to include the capacity to perform DNA isolation from a range of samples. However, at the launch of the product, it became clear that this feature was not included, and the current version of the equipment (November 2022) is only able to prepare ONT sequencing libraries which requires high-quality DNA as input.

At In-8, the VolTrax V2 system was tested using DNA extracted using the PDQeX and the Purelink Microbiome DNA isolation kit (Invitrogen) from faeces. While the system produced several failed runs for non-apparent reasons, specifically for multiplex samples, single-plex library preparation performed reasonably well. Samples were prepared using the VSK-002 kit. Quality metrics of the generated data showed a better performance using the Purelink Microbiome DNA isolation kit, see figure 2. Total reads, total bases, mean fragment length and mean quality were significantly better when the higher quality DNA was used for input.

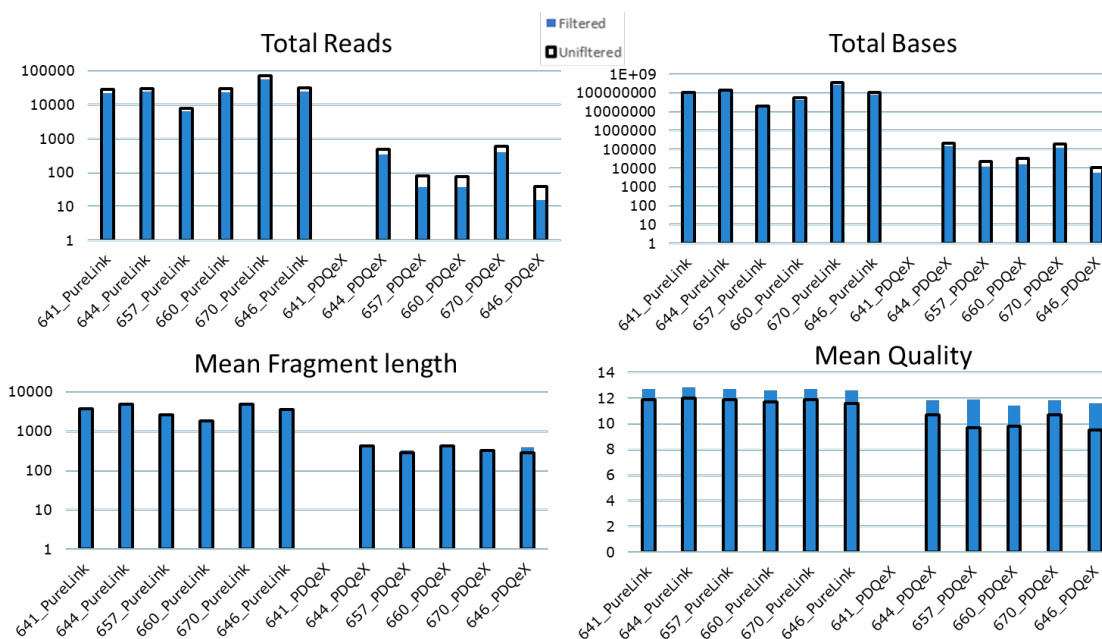


Figure 2. Faecal samples sequenced on VolTrax V2, comparing Purelink and PDQeX isolated DNA.

In-6, initially tested automated library preparation using the VolTrax V2 on phage lambda control DNA (Oxford Nanopore Technologies), using two VolTrax sequencing kit versions: VSK-VSK002 and VSK-VSK003. The libraries were sequenced on the flongle R9.4.1 flowcell. While the library prepared with kit version VSK-VSK002 yielded adequate results, the VSK-VSK003 performed poorly, with low poor occupancy indicating a failed library preparation (data not shown). This could also be attributed to performance issues with Flongle flow cells, as these showed rapid degradation of pore counts after library loading (data not shown). However, all subsequent experiments on the VolTrax V2 failed, with both VolTrax library preparation kits yielding low read counts (Table 5). Finally, when the V2b version of the VolTrax device was used in combination with the updated library preparation kit, a single



sequencing run on a chicken faeces metagenomics sample spiked with the Zymo GMS mock community (cfr. Deliverable D-JRP12-1.1, <https://zenodo.org/record/7429361>) produced adequate results.

Table 5. Outcome of VolTrax V2 and V2b tests performed

Sample	DNA extraction and purification method	Library preparation	Voltrax version	Flow cell version	Total sequenced bases
Lambda control DNA	/	VSK-VSK002	2	Flongle R9.4.1	929.59 Mbp
Lambda control DNA	/	VSK-VSK003	2	Flongle R9.4.1	2.97 Mbp
Zymo GMS	Modified Zymo QuickDNA HMW magbead kit	VSK-VSK002	2	MinION R9.4.1	13.96 Mbp
Zymo GMS	Modified Zymo QuickDNA HMW magbead kit	VSK-VSK002	2	MinION R9.4.1	22.52 Mbp
Zymo GMS	Modified Zymo QuickDNA HMW magbead kit	VSK-VSK003	2	MinION R9.4.1	63.27 Mbp
Chicken faecal sample + zymo GMS spike-in	On-site DNA extraction method (appendix 2)	VSK-VSK004	2b	MinION R9.4.1	14 Gbp

4. Tests performed for on-site long-read sequencing of sample matrices

4.1. On-site diagnostic tests for mastitis milk

An on-site diagnostic test of bovine milk from mastitis suspected cows was performed at the Aeres agricultural school in Dronten, the Netherlands. The tests were performed by veterinarians with no molecular diagnostic training, under supervision of trained In-8 staff. The purpose of the test was to detect potential mastitis causing agents and AMR genes, and to compare these to standard culture methods. As culture-dependent methods have a long turnover time, these data are currently not available yet. DNA was isolated using the PDQeX method as described in appendix 2, from 8 high-cell count milk samples. DNA was prepared using the Rapid barcoding kit SQK-RBK004. Sequencing was done with flowcell Minlon R9.4.1. As the complete DNA isolation and sequencing was performed on-site within 8 hours, the amount of data is limited (table 6) and the limit of detection for pathogen and AMR identification will need to be determined against further diagnostic samples.

Table 6. Quality metrics of on-site DNA sequencing of mastitis milk.

Sample	Total bases	Total reads	Mean read length	Mean read quality
A	1406357	5373	261.7	9.3
B	5349157	9814	545.1	10.0
C	13545212	23234	583.0	10.1
D	4077725	7928	514.3	9.8
E	12088709	23709	509.9	9.9
F	2237300	11509	194.4	9.4
G	13877523	20649	672.1	9.9
H	936161	3666	255.4	9.2



Bacterial species were identified using KMA. Due to the limited size of the reads, a large proportion could not be assigned to specific species. The top 3 of each milk sample is shown in table 7. *E. coli* and *Actinoalloteichus* sp. were present in all samples at relatively high abundance. Although these bacteria are both found in non-mastitis milk, both can also cause mastitis symptoms. Comparison with the culture-dependent detection is essential, when these data become available.

Table 7. Relative abundance of identified bacterial species in mastitis milk.

Bacteria	Sample							
	A	B	C	D	E	F	G	H
<i>Azorhizobium caulinodans</i>					4.2 E-05	8.7 E-05	4.8 E-05	
<i>Escherichia coli</i>	0.13	0.03	0.06	0.03	0.05	0.08	0.02	0.02
<i>Listeria monocytogenes</i>	1.9 E-04							
<i>Rhodobaca barguzinensis</i>								1.3 E-03
<i>Actinoalloteichus</i> sp.	0.01	0.06	0.03	0.03	0.03	4.9 E-03	0.04	4.3 E-03
<i>Clostridium botulinum</i>		1.0 E-03	3.8 E-04	1.2 E-04				

AMR genes were identified using KMA. As whole genomes were sequenced, it was to be expected that only a small proportion of reads would include AMR-genes. The top 3 genes of each sample is shown in table 8. This shows that β -lactamase genes were present in all samples, but the species in which these genes are present could not be assigned. Furthermore, resistance against aminoglycoside, lincosamide and vancomycin were detected in some samples.

Table 8. Relative abundance of identified AMR-genes in mastitis milk.

AMR gene	Sample							
	A	B	C	D	E	F	G	H
<i>aadA11</i>							6.3 E-04	
<i>aadA23</i>	7.4 E-04		7.3 E-04	7.6 E-04		3.5 E-04		1.1 E-03
<i>aadA24</i>		8.2 E-04						
<i>aph(6)-Ia</i>	1.9 E-04							
<i>bla_{OXA-315}</i>				5.1 E-04	3.4 E-04	1.7 E-04		5.5 E-04
<i>bla_{OXA-443}</i>					7.1 E-04		6.3 E-04	
<i>bla_{OXA-455}</i>	1.5 E-03	1.8 E-03	2.4 E-03	2.0 E-03	3.7 E-03	2.3 E-03	3.2 E-03	1.6 E-03
<i>Inu(E)</i>		3.1 E-04						
<i>VanHOX</i>			4.3 E-04					
Total AMR	6.5 E-03	1.5 E-02	1.8 E-02	1.8 E-02	1.9 E-02	6.9 E-03	2.1 E-02	6.6 E-03

4.2. Mock on-site diagnostic tests from chicken faeces

Institute 6 performed mock on-site diagnostic tests for the detection of pathogens (represented by a spiked mock defined microbial community (GMS, cfr Deliverable D-JRP12-1.1, <https://zenodo.org/record/7429361>) in chicken faeces. The test included the DNA extraction protocol developed for on-site usage (cfr. appendix 2) requiring a vortex, magnet, hula mixer and the Bento Bio Pro, which are all feasible to be used on-site. To prepare a library from the DNA extract, the VolTrax V2b device was used, requiring a laptop with USB-C outlet. Alternatively, the VolTrax can be replaced by the rapid sequencing kit (SQK-RAD004, stored at -20 °C) or the field sequencing kit (SQK-LRK001, which can be stored at ambient temperatures) to possibly reduce reagent costs and cold-chain requirements. The library was sequenced on a MiniON R9.4.1 flowcell on the Gridion device (Oxford Nanopore Technologies) and basecalling was performed afterwards on a high-performance cluster,



using guppy_gpu v5.0.7. For on-site applicability, sequencing can be performed on a Mk1C device, with real-time basecalling enabled. A quality check on the sequencing run was done with NanoPlot v1.36.2. Data was analysed using the KMA tool (cfr Deliverable D-JRP12-2.1+2.2, <https://zenodo.org/record/7433258>). The results demonstrated that the workflow provides satisfactory long-read sequencing data (N50 ~8kb, mean qscore ~12 and >2 million reads) (Table 9), allowing the detection of the spiked bacterial species at least to the same level as obtained using conventional lab protocols (cfr. Deliverable D-JRP12-1.1, <https://zenodo.org/record/7429361>).

Table 9. KMA classification off on-site method compared to the conventional Zymo QuickDNA HMW magbead + MetaPolyzyme method (see D-JRP12-1.1, <https://zenodo.org/record/7429361>)

Species in GMS	Relat. abund.	Nbr of cells	Gram	Sample	Pure Zymo GMS	Zymo GMS in chicken faeces	Zymo GMS in chicken faeces
				DNA extraction method	Zymo QuickDNA HMW magbead + MetaPolyzyme	Zymo QuickDNA HMW magbead + MetaPolyzyme	On-site method
				Library preparation	SQK-LSK109	SQK-LSK109	VSK-VSK004
<i>Escherichia coli</i>	14%	3×10^7	-		+	+	+
<i>Faecalibacterium prausnitzii</i>	14%	3×10^7	+		+/-	+/-	+/-
<i>Veillonella rogosae</i>	14%	3×10^7	-		+	+	+
<i>Roseburia hominis</i>	14%	3×10^7	+/-		+	+	+
<i>Bacteroides fragilis</i>	14%	3×10^7	-		+	+	+
<i>Prevotella corporis</i>	6%	1×10^7	-		+	+	+
<i>Bifidobacterium adolescentis</i>	6%	1×10^7	+		+	-	+
<i>Fusobacterium nucleatum</i>	6%	1×10^7	-		+	+	+
<i>Lactobacillus fermentum</i>	6%	1×10^7	+		+	+	+
<i>Clostridioides/dium difficile</i>	1.50%	3×10^6	+		+	+	+
<i>Akkermansia muciniphila</i>	1.50%	3×10^6	-		+	+	+
<i>Methanobrevibacter smithii</i>	0.10%	2×10^5	+		+	-	+
<i>Salmonella enterica</i>	0.01%	2×10^4	-		+/-	-	-
<i>Enterococcus faecalis</i>	0.001%	2×10^3	+		-	-	-
<i>Clostridium perfringens</i>	0.0001%	2×10^2	+		-	-	-

4.3. On-site diagnostic tests from pig faeces

At institute 7, DNA yields from the on-site extractions (from faeces and soil using Quick-DNA HMW MagBead Kit (D6060, Zymo Research) on the BentoLab device) were used for ONT sequencing on-site. DNA Libraries were prepared using two protocols: Genomic DNA by Ligation (SQK-LSK109), and Field Sequencing Kit (SQK-LRK001). All raw outputs (Fast5) were basecalled using an in-house built GPU-based laptop with the Guppy-basecaller pipeline and high accuracy basecalling option (please see WP2 deliverable for the pipeline details, <https://zenodo.org/record/7433258>). All resulted Fastq files were fed into KMA for bacterial taxa and AMR identifications. From the faecal sample, approximately 65000 reads were assigned to 275 bacterial species and 12 AMR genes using library preparation kit LSK109. The gut microbiome bacteria dominated the most abundant bacterial taxa, Firmicutes members were the dominant bacterial phylum. *Lactobacillus* species were the most abundant bacteria, followed by members in *Faecalibacterium*, *Alistipes*, *Prevotella* and *Bifidobacterium*. Those results agree with the bacterial taxa that typically dominate pig gut microbiomes.

Only 1091 reads were assigned to 13 bacterial taxa using the Field Sequencing kit LRK001. This indicates that LSK109 ligation kit performs markedly better than the quick Field sequencing kit LRK001. *S. aureus* species was one of the abundant bacterial taxa in the microbiome analyses from the tested



faeces as it was added as a spiked-in control.

4.4. *On-site diagnostic tests from bootsocks in poultry farms*

Institute 3 prepared genomic libraries using the Rapid Barcoding kit (SQK-RBK110.96) but only individual samples were analysed per sequencing run using the relatively small Flongle flow cells. To overcome the low DNA input we prepared 3 libraries per sample, using the same barcode, and pooled these together before the clean-up step. According to the fast protocol, sequencing ran just 4 hours with fast basecalling. Sequencing runs were monitored in real time by using Epi2me software and then analysed by KMA in the lab. The output (total bp produced) was generally lower than the expected most probably caused by different factors: the low number of sequencing pores active in the Flongle flowcells, the low input DNA, the rapid protocol (less efficient than others i.e. ligation kit), and the short time set for sequencing. Despite this, sequencing by MinION allowed to detect the presence of *Campylobacter* in all 6 bootsocks, data confirmed by culture, PCR and KMA analysis (Figure 3).

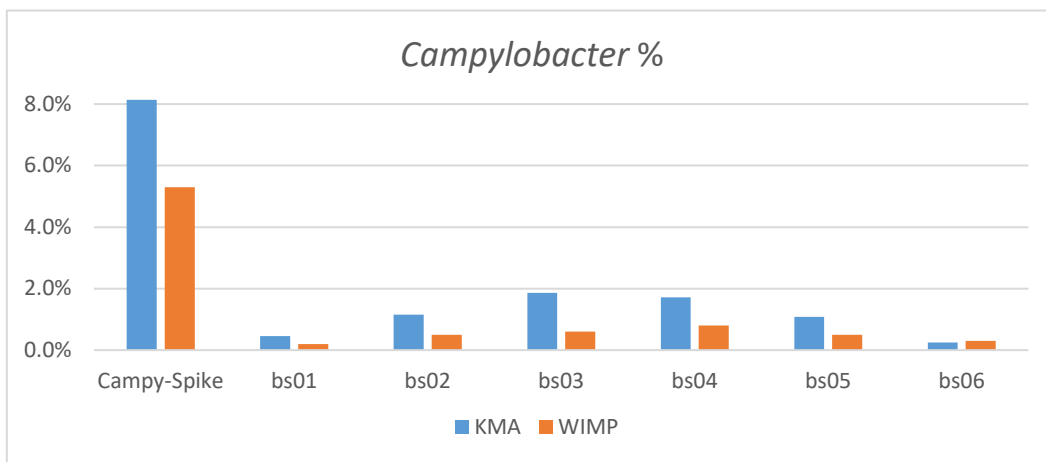


Figure 3. Percentage of reads detected by KMA and WIMP (Epi2me) for *Campylobacter* identification in bootsock samples.

Conversely, *Salmonella* was recognised by WIMP (Whats In My Pot) pipeline but the presence of the pathogen was not confirmed by PCR, nor by culture, and just very few reads (1 or 2) were retrieved by KMA analysis in bootsock 01, 04 and 05 while no reads in bootsock 02, 03 and 06 (Figure 4).

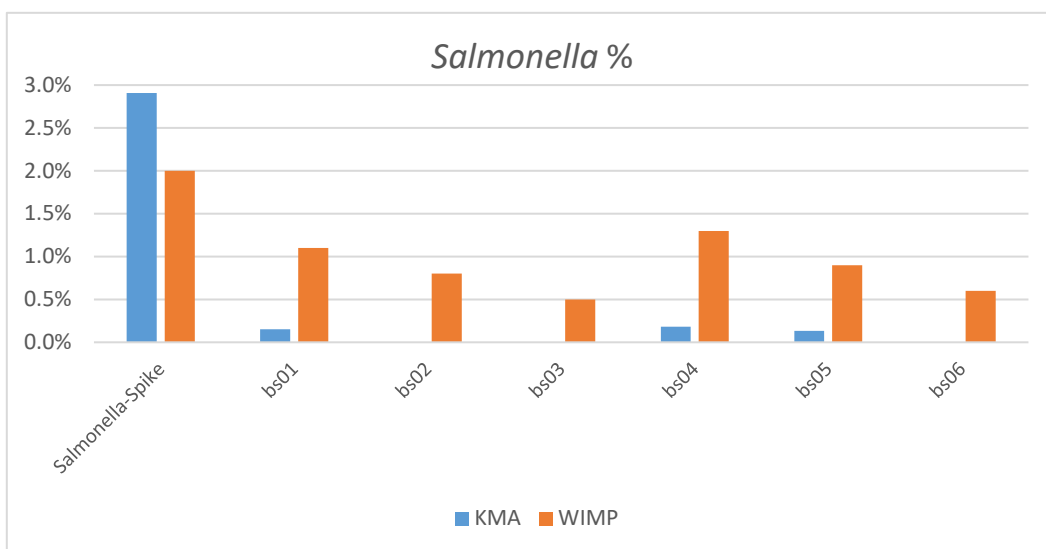


Figure 4. Percentage of reads detected by KMA and WIMP (Epi2me) for *Salmonella* identification in bootsock samples.



The reads of *Salmonella* identified by WIMP should be further investigated, for sensitivity and specificity. We finally investigated the presence of AMR genes in the samples by using two methods for analysis, Epi2Me and KMA, detail in deliverable WP2 (<https://zenodo.org/record/7433258>). In table 10, summarizes the AMR gene content results.

Table 10. AMR results obtained by Fastq Antimicrobial Resistance (FAR) tool (Epi2me), and KMA/Resfinder. In the last row the list of AMR genes found by both methods.

	bs01	bs02	bs03	bs04	bs05	bs06
Epi2me	<i>cat, tetW</i>	<i>mdtL</i>		<i>ermF, tetX, cpxA</i>		<i>tetW</i>
KMA	<i>msr(A)</i>		<i>tet(W)</i>	<i>tet(32)</i>		
KMA and Epi2me (Concordance)	<i>tetK</i>	<i>ant(6)-Ia, tet(O/32/O), tet(Q), tet(W)</i>	<i>erm(B), tet(O/32/O), tet(Q)</i>	<i>ant(6)-Ia, tet(O), tet(W)</i>	<i>mphC, tetQ, tet32, ANT(6)-Ib</i>	

5. Concluding remarks

DNA sequencing can be performed using many different techniques, but all rely on the isolation of a clean DNA sample in order to perform adequately. While again many different techniques are available for this isolation, often these require specialised equipment which is not suitable for on-site analysis. In the preparation of FARMED, this challenge was recognised and as such, a literature review was conducted to identify promising techniques for on-site usage.

Several DNA isolation techniques have been tested for on-site, or mock on-site, usage during the project. Here, specific thought was given to use techniques that would require the least amount of equipment to bring into the field, and which produces the least amounts of hazardous waste. The type of sample matrix, water, milk, or faeces, proved to be increasingly difficult and even the host species from which faeces was collected, was shown during the project to influence the yield or quality of DNA that could be isolated.

At the initiation of the FARMED project, it was expected that DNA isolations, as well as preparation of sequence libraries, could be carried out on the VolTrax V2 device by Oxford Nanopore Technologies. Upon its release it became clear that the current version of the device does not have this capacity to isolate DNA and an alternative was found in the PDQeX device by MicroGEM, for which protocols for isolation from faeces and milk have been described. Thus, the VolTrax V2 device was currently only tested for preparation of sequence libraries, for which it was also used in the setting of one of the on-site tests.

On-site, or mock on-site, tests have successfully been conducted by four partners of the consortium. In most cases, samples that were tested on-site had been spiked with known pathogens or tested with additional methods in order to determine the accuracy of the analysis. While the expected pathogens could mostly be identified, both during laboratory testing and the on-site tests, the detection of AMR genes requires a deeper level of sequencing that could not be reached for all samples. Furthermore, no clear cut-offs are currently defined in terms of the limit of detection for this type of analysis, which is a limitation that future projects will need to address.