



Deliverable
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Workpackage 3
Classification of pig farm
compartments according to
their role in the
epidemiology of
***Clostridioides difficile* (task**
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D-JRP15-FED-AMR-WP3.3

D-JRP15–FED-AMR-WP3.3 Classification of pig farm compartments according to their role in the epidemiology of *Clostridioides difficile*

Description of the task

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Description of the task: The aim of this task is to evaluate *C. difficile* strains/AMR dissemination between humans, animals and the environment using the pig farm as proof of concept. *C. difficile* prevalence and variety regarding ribotypes and AMR profile will be described in the different compartments: the presence of zoonotic *C. difficile* ribotypes; presence of a variety of ribotypes; presence of *C. difficile* toxin-genes (*tcdA*, *tcdB*, *cdt*) and presence of AMR genes with possibility for exchange of these genes between different *C. difficile* ribotypes and environments. Based on this information the compartments will be classified according to their possible role in *C. difficile* epidemiology, e.g. a compartment that contains a specific ribotype in higher numbers may be critical for dissemination. We expect to identify transmission networks for *C. difficile* and AMR dissemination and respective critical compartments.

Description of deliverable

Abstract

In order to assess *C. difficile*/AMR dissemination between humans, animals and the environment, studies were conducted on samples from 4 countries (2 HOALs Portugal, Austria, 1 agricultural facility Estonia, single compartments Ireland). The results obtained allowed to perform a classification of pig farm compartments according to their role in the epidemiology of *C. difficile*, which was identified in all the following ecosystems: pig barn (faeces), manure (pig), wild animals, soil (agricultural fields), river water, ground water, wastewater treatment plant and air. In addition, in each HOAL, a different toxigenic *C. difficile* RT (RT033, RT078 and RT049, respectively) was dominant, reflecting well-established transmission chains and a resilient source.

Overall, the results obtained from the 3 HOALs/agricultural facility showing the presence of dominant clones in compartments associated with the pig production unit suggest a transmission chain involving these animals. They contribute to unveiling the role played by animal and environmental reservoirs within the *C. difficile* epidemiology.

HOAL Portugal

Scientific outputs from this task are already published in detail in two scientific papers with



peer-review.

Alves F, Nunes A, Castro R, Sequeira A, Moreira O, Matias R, Rodrigues JC, Silveira L, Gomes JP, Oleastro M. (2022) Assessment of the Transmission Dynamics of *Clostridioides difficile* in a Farm Environment Reveals the Presence of a New Toxigenic Strain Connected to Swine Production. *Front Microbiol.* 2022 Apr 14;13:858310. doi: 10.3389/fmicb.2022.858310 <https://zenodo.org/record/7446535#.Y5xSj1GZND8>

Alves F, Cano M, Brondani G, Nunes A, Oleastro M. (2022) Airborne spores' dissemination of a swine associated *Clostridioides difficile* clone. *Anaerobe.* 2022 Sep 29;78:102651. doi: 10.1016/j.anaerobe.2022.10265 <https://zenodo.org/record/7446535#.Y5xSj1GZND8>

A predominant clone of RT033 was found in samples from 4 compartments connected to the pig production unit, compartments 1 pig barn, pooled pig faeces, compartment 2 manure, compartment 3 soil, compartment 10 wastewater treatment plant (Tab. 1). Core-genome SNV-based analysis supported a clonal transmission between the compartments. The RT033 HOAL clone differed from known RT033 in regard to toxin genes and represents a new toxigenic strain. The phylogenetic positioning of this clone was clearly distinct from the classical RT033 cluster, being the first report of a toxigenic RT033 clone. This clone displayed a unique combination of genetic elements that may contribute to host tropism, environmental dissemination and maintenance.

Table 1: Overview of the *C. difficile* prevalence and RTs in compartments of the HOAL Portugal

Compartments	Ecosystem	<i>C. difficile</i> prevalence	RTs
Compartment 1	Pig barn; faeces		
	Fattening pigs	0% (0/18)	Sows RT033 Piglets RT033
	Reproduction sows Piglets	24% (6/25) 60.5% (26/43)	
Compartment 2	Manure (pig)		
	Swine manure Swine waste cesspools	66.7% (4/6) 73% (8/11)	RT033 RT033
Compartment 3	Soil: agricultural fields		
	Fertilized agricultural soil	100% (6/6)	RT033
	Non-fertilized agricultural soil	66.7% (6/9)	RT033
	Non-agricultural soil (Forest) Non-agricultural soil (Meadow)	22.2% (2/9) 55.5% (5/9)	RT033, RT027 RT033, RT720, RT126
Compartment 4	Crop	-	
Compartment 5	Field drainage	0% (0/1)	



Compartment 6	Surface, river water	0% (0/1)	
Compartment 7	Drinking water, ground water	0% (0/1)	
Compartment 8	Feed	-	
Compartment 9	Farmers	0% (0/17*)	
Compartment 10	Wastewater treatment plant	100% (4/4)	RT033 RT005 RT071 RT643
Compartment 11	Wild Animals	-	

* These numbers refer to two sampling points from the same farmers.

In an additional study air samples were collected in three different maternity units within a swine reproduction unit, where 100% of the air samples from the farrowing (2/2) and weaning (2/2) units were positive. All Isolates of the *C. difficile* positive samples belonged to the toxigenic RT033 clone which denotes a well-established transmission network and adds the possibility of airborne dissemination of spores as an additional contribution to bacterial spread.

HOAL Austria

In the Austrian HOAL three compartments were found to be positive for *C. difficile*. The obtained isolates all belonged to the toxigenic RT078 (Tab. 2). Three wastewater samples (2 inlet samples, one outlet sample) yielded RT078 isolates. Further, three manure samples and one wild animal feces sample collected on a farmer's field (compost) contained *C. difficile* RT078. With a cluster threshold of 6 alleles for cgMLST four of the Isolates (2 manure isolates, one wastewater outlet isolate and the wildlife isolate) formed a cluster across the three compartments.

Table 2: Overview of the *C. difficile* prevalence and RTs in compartments of the HOAL Austria

Compartments	Ecosystem	<i>C. difficile</i> prevalence	RTs
Compartment 1	Pig barn; faeces	-	
Compartment 2	Manure (pig)	37.5% (3/8)	RT078
Compartment 3	Soil: agricultural fields	0% (0/35)	



Compartment 4	Crop	-	
Compartment 5	Field drainage	0% (0/4)	
Compartment 6	Surface, river water	0% (0/4)	
Compartment 7	Drinking water, ground water	0% (0/4)	
Compartment 8	Feed	0% (0/5)	
Compartment 9	Farmers	-	
Compartment 10	Wastewater treatment plant	37.5% (3/8)	RT078
Compartment 11	Wild Animals	7.7% (1/13)	RT078

Agricultural facility Estonia

In the Estonian facility toxigenic *C. difficile* RT049 was found to be the dominating ribotype (Tab. 3). With a cluster threshold of 6 alleles for cgMLST, isolates from 12 samples formed a cluster. These samples belonged to the compartments 1 Pig barn, pooled pig faeces, compartment 2 Manure, compartment 3 soil, agricultural fields, meadow soil and forest soil, compartment 10 Wastewater treatment plant, waste water tank.

Table 3: Overview of the *C. difficile* prevalence and RTs in compartments of the agricultural facility Estonia

Compartments	Ecosystem	<i>C. difficile</i> prevalence	RTs
Compartment 1	Pig barn; faeces Pooled pig faeces	17% (4/24)	RT049 RTAI-83
Compartment 2	Manure (pig)	50% (1/2)	RT049
Compartment 3	Soil Agricultural fields Meadow soil Forest soil	64% (18/28)	RT049 RT002/2 RT639 RT051 RT012 RT005 RT010
Compartment 4	Crop	-	



Compartment 5	Field drainage	-	
Compartment 6	Surface, river water River water	50% (1/2)	RT078
Compartment 7	Drinking water, ground water Ground water	33% (1/3)	RT078
Compartment 8	Feed	-	
Compartment 9	Farmers	0% (0/21)	
Compartment 10	Wastewater treatment plant Wastewater	100% (3/3)	RT049 New RT
Compartment 11	Wild Animals		

Sampling site Ireland

From the sampling site Ireland 10 faecal samples from deer (compartment 11) tested negative for *C. difficile* while 3 of 4 samples from a waste water treatment plant (compartment 10) yielded *C. difficile* isolates of different ribotypes, both toxigenic and non-toxigenic (RT039, RT012, RT078, RT054 and 2 new RTs).

Results

First, it is striking that each of the tested 3 HOALs/agricultural facility has one distinct RT that is dominantly detectable in almost all compartments that tested positive. That is, a new toxigenic RT033 clone in Portugal (Tab.1), the well known hypervirulent and zoonotic RT078 in the HOAL Austria (Tab. 2) and the toxigenic RT049 in the Estonian agricultural facility (Tab. 3). Another noteworthy finding is that some, though not all isolates, of these dominant RTs in each of the sites are genetically so closely related that it can be assumed that they are distributed in the respective HOAL network. RT078 was detected in three sampling sites - Austria, Ireland and Estonia - highlighting its wide distribution and importance as a potential zoonotic agent.

Positive compartments and prevalence

Pig Barn

Compartment 1 (pig barn; faeces) samples were collected from 2 sites. The findings from the HOAL Portugal demonstrates the age dependency of *C. difficile* prevalence with a marked higher prevalence in piglets than in older animals (Tab. 1). Besides RT033 no other RTs were found in this compartment. In addition, the possibility of airborne dissemination of RT033 could be demonstrated here. In the Estonian facility *C. difficile* RT049 was found in pooled pig faeces with a prevalence of 17% indicating a mixed or older animal population. Beside the dominating RT049 another RT was also detected (Tab. 3).

Manure



Manure was positive for *C. difficile* in 3 of 3 tested sites (Portugal, Austria, Estonia) and in case faecal samples were collected at the same site, manure showed the higher percentage of positive samples (Tab. 1, Tab. 3) only the repective dominating RT was found in this compartment in all of the three sites.

Soil

In compartment 3, *C. difficile* was detected in two of the three sites investigated (Tab. 4). The compartment was divided into different subcategories in two of the three sites investigated (Tab. 1, Tab. 3), with a relatively high overall rate of *C. difficile*-positive samples. It is also noteworthy that in two of the sampling sites the diversity of detected ribotypes was quite high; besides the dominant RT, three other RTs were found in Portugal, while seven different RTs were detected in Estonia.

Wastewater

In compartment 10, *C. difficile* was also detected in all three sites investigated (Tab. 4). In addition, wastewater samples from Ireland were examined. Here, too, a rather high rate of *C. difficile*-positive samples was found. The diversity of detected ribotypes was relatively high in three sites; four different RTs were found in Portugal, while two different RTs were detected in Estonia and six in Ireland.

Table 4: Summary of results concerning compartments of three sites (HOALs Portugal and Austria and agricultural facility Estonia)

Compartments	Ecosystem	Sites tested	<i>C. difficile</i> presence
Compartment 1	Pig barn; faeces, air*	2	2
Compartment 2	Manure (pig)	3	3
Compartment 3	Soil: agricultural fields	3	2
Compartment 4	Crop	0	
Compartment 5	Field drainage	3	0
Compartment 6	Surface, river water	3	1
Compartment 7	Drinking water, ground water	3	1
Compartment 8	Feed	0	0
Compartment 9	Farmers	2	0



Compartment 10	Wastewater treatment plant	4	4
Compartment 11	Wild Animals	2	1

* additional air sampling in HOAL Portugal

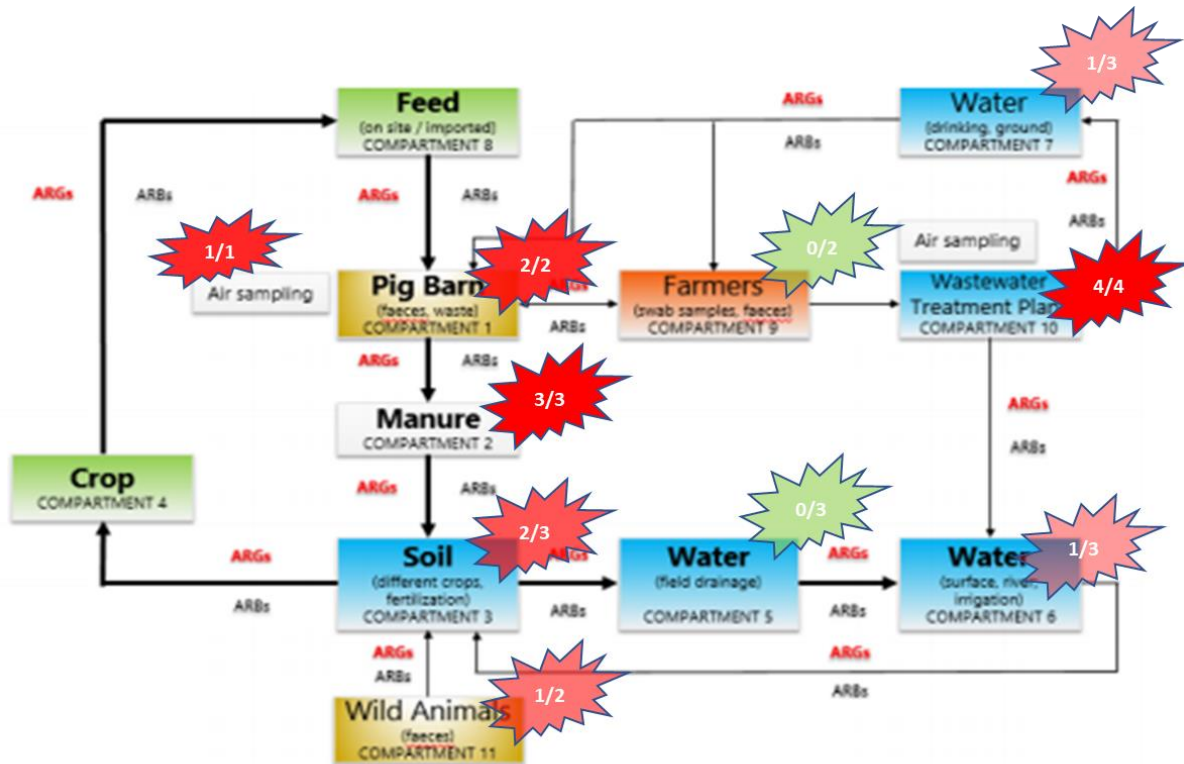


Fig.1. Potential pathways for ARG dissemination over environmental ecosystem barriers. Bold letters/arrows: monitored compartments and pathways for ARG. ARBs: antimicrobial resistant bacteria. Animal compartments: gold. Human compartments: red. Plant-associated compartments: green. Genuine environmental compartments: blue.

Figure 1: Adapted figure from the FED-AMR proposal. Graphical representation of the results from Table 4. Above the compartments is indicated how many tested positive and how many sites were tested.

Possible role of the compartments in the dissemination of *C. difficile*, conclusions and classification

The studies show that *C. difficile* can spread (clonally) across the different compartments, although the results obtained here do not allow a clear conclusion on the possible direction of spread. However, it is noteworthy that there is a greater diversity in terms of RTs in the two compartments soil and wastewater, while fecal and manure samples in individual facilities may indicate the dominant RT. This rather suggests a more unidirectional spreading out of the animal compartments into the environment. On the other hand, the soil and wastewater compartments seem to act as a reservoir for different input pathways beyond the animal compartments. Of particular interest is the possibility that *C. difficile* is transferred back to



animal compartments via groundwater (compartment 6) and surface water (compartment 7) (Fig. 1). While they were not positive for *C. difficile* to any great extent, the results from Estonia (Tab. 3) suggest this possibility.

In any case, the introduction of antibiotics or other substances that promote the development of antimicrobial resistance in *C. difficile* into any of the compartments can have an impact on the other compartments. Antibiotic applications on animals may be particularly sensitive in this respect. Interestingly, the farmer's samples negative results for *C. difficile* have shown an apparently low risk of transmission to humans, but the number of samples was small and the literature shows exactly such events.

From the results presented here, we conclude that the manure compartment in particular is suitable for detecting the dominant RT on the farm, while the wastewater compartment provides an overview of other circulating RTs.