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RESEARCH ARTICLE

Strategy to develop and validate digital droplet PCR methods for global antimicrobial resistance wastewater surveillance

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Abstract

According to World Health Organization (WHO), antimicrobial resistance (AMR) is currently one of the world's top 10 health threats, causing infections to become difficult or impossible to treat, increasing the risk of disease spread, severe illness, disability, and death. Accurate surveillance is a key component in the fight against AMR. Wastewater is progressively becoming a new player in AMR surveillance, with the promise of a cost-effective real-time tracking of global AMR profiles in specific regions. One of the most useful analytical methods for wastewater surveillance is currently based on real-time PCR (qPCR) and digital droplet PCR (ddPCR) technologies.

As stated in the EU Wastewater Treatment Directive proposal, methodological standardization, including a workflow for method development and validation, will play a crucial role in global monitoring of AMR in wastewater. However, according to our knowledge, there are currently no qPCR and ddPCR methods for AMR surveillance available that have been validated according to international standard performance criteria. Therefore, this study proposes a workflow for the development and validation of PCR-based methods for a harmonized and global AMR surveillance, including the construction of specific sequence databases and microbial collections for an efficient method development and method specificity evaluation. Following this strategy, we have developed and validated four duplex ddPCR methods responding to international standard performance criteria, focusing on seven AMR genes (ARG's), including extended spectrum beta-lactam ($bla_{CTX\text{-}M}$), carbapenem

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(*bla*$_{KPC\text{-}2/3}$), tetracycline (*tet(M)*), erythromycin (*erm(B)*), vancomycin (*vanA*), sulfonamide (*sul2*), and aminoglycoside (*aac(3)-IV*), as well as one indicator of antibiotic (multi-) resistance and horizontal gene transfer, named the class I integron (*intl1*). The performance of these ddPCR methods was successfully assessed for their specificity, as no false-positive and false-negative results were observed. These ddPCR methods were also considered to be highly sensitive as showing a limit of detection below 25 copies of the targets. In addition, their applicability was confirmed using 14 wastewater samples collected from two Belgian water resource recovery facilities. The proposed study represents therefore a step forward to reinforce method harmonization in the context of the global AMR surveillance in wastewater.

Practitioner Points:

- In the context of wastewater surveillance, no PCR-based methods for global AMR monitoring are currently validated according to international standards.
- Consequently, we propose a workflow to develop and validate PCR-based methods for a harmonized and global AMR surveillance.
- This workflow resulted here in four duplex ddPCR methods targeting seven ARGs and one general indicator for mobilizable resistance genes.
- The applicability of these validated ddPCR methods was confirmed on 14 wastewater samples from two Belgian water resource recovery facilities.

KEYWORDS

antimicrobial resistance genes, ddPCR, detection, quantification, surveillance, wastewater

INTRODUCTION

Antimicrobial resistance (AMR) poses a significant threat to human, animal, and environmental health. The overuse and misuse of antibiotics have fueled the emergence and dissemination of microbes carrying multiple AMR genes (ARG's) (O'Neill, 2014). AMR currently ranks among the top 10 worldwide global health threats and is causing infections to become difficult or impossible to treat, increasing the risk of disease spread, severe illness, disability, and death (European Public Health Alliance, 2022; World Health Organization, 2022). In 2022, 4.95 million deaths were associated with AMR leading to ineffective treatment. If no action is taken, the devastating impact of AMR is expected to increase. By 2050, it is estimated that the number of deaths attributable to AMR could escalate to the alarming rate of one person dying every 3 s (Review on Antimicrobial Resistance, 2016).

To address AMR effectively, to prioritize actions and to develop new relevant antimicrobial drugs, a robust AMR surveillance is crucial (European Public Health Alliance, 2022; Tiwari et al., 2022; World Health Organization, 2022). National and international AMR surveillance initiatives are currently focusing on a few specific pathogens in clinical settings, following the WHO priorities (WHO, 2017). Moreover, large scientific networks are sharing AMR data as illustrated by WHO collaborating with 126 countries, territories, and areas for the AMR surveillance through the Global Antimicrobial Resistance and Use Surveillance System (GLASS) project (World Health Organization, 2022). However, as mentioned by Chau et al., 2022, "current surveillance may be limited by the dependence on individual-level sampling, frequently affected by selection bias towards healthcare-associated settings. Moreover, data collection typically focuses exclusively on a subset of cultivable species and on susceptibility phenotypes rather than AMR genotypes. Consequently, the monitoring of high-risk AMR-associated clones and specific AMR-associated genetic determinants is hampered" (Chau et al., 2022).

While these national and international AMR surveillance initiatives provide crucial information, they often require a large number of patients and focus solely on

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diseases associated with clinical signs and symptoms leading to hospitalization, overlooking the general prevalence of AMR (Tiwari et al., 2022). Wastewater-based epidemiology, using wastewater data to generate information on human populations at the community scale, has definitely proved its efficiency for global monitoring of SARS-CoV-2 and poliovirus (Hassard et al., 2023; Larsson et al., 2023). In addition, for wastewater sampling, a threshold related to individual privacy is still under discussion and has not yet been defined. It is currently widely accepted within the scientific community that for sampling at urban wastewater treatment plants gathering wastewater from tens of thousands, hundreds of thousands, or even millions of citizens; no connections can be made with individual data. Therefore, legal and ethical concerns related to individual privacy are minimal by using this population-level sampling approach (Doorn, 2022; Sambaza & Naicker, 2023; Tiwari et al., 2022). Wastewater to strengthen AMR surveillance has also gained attention as a cost-effective approach, covering the global AMR profile at the population level from a defined region and enabling real-time tracking of AMR profiles. A step towards a global monitoring of AMR in wastewater in Europe was taken by the proposition of revision of the Urban Waste Water Treatment Directive in order to allow regular monitoring of AMR in water resource recovery facilities (WRRF) (European Commission, 2022; jpiamr, n.d.; Larsson et al., 2023). However, for such global implementation, some challenges currently remain, regarding, for instance, the selection of the AMR targets and method performance criteria with a harmonized validation workflow. Indeed, heterogenous study designs and methods may lead to differences in outcomes and data interpretations within and between EU countries. To minimize it, the methodology standardization is therefore essential. To this end, a workflow for the development and validation of detection methods as well as the establishment of minimum performance criteria for methods used for the wastewater surveillance is needed for harmonizing and facilitating comparison of results between countries (Chau et al., 2022; Larsson et al., 2023).

For wastewater analysis, PCR-based methods are commonly used (Home|EU-WISH, n.d.; Adams et al., 2024; CDC, 2023a; CDC, 2023b; Gerdes et al., 2016; Van Poelvoorde et al., 2021 ; Van Poelvoorde et al., 2023). Notably, digital droplet PCR (ddPCR) offers advantages such as the simultaneous detection and absolute quantification without dependency on reference materials, the high tolerance to PCR inhibitors, and the direct expression of the generated data as the copy number of the target of interest (Van Poelvoorde et al., 2021; Van Poelvoorde et al., 2023). To analyze ARG's in water samples, only a few studies using ddPCR methods are currently published. In these studies, different sets of target genes were selected based on local epidemiology, including *aadA, eis, ermB, tetA, tetC, tetM, tetQ, tetW, sul1, strA, strB, mecA, vanA,* bla_{TEM}*,* bla_{OXA}*,* bla_{KPC}*, dfr13, qnrS, qnrA, gyrA, gyrB, acrD, qacF, katG, rpoB, embB, pncA, rrs, atpE, ethR*, and *intl1*; the class I integron used as general indicator for mobilizable resistance genes (Amarasiri et al., 2022; Mtetwa et al., 2021; Rumky et al., 2022; Wang et al., 2018). These few published methods show the diversity of targets that can be used. However, none of these PCR assays are validated according to international performance criteria requirements (European Commission, n.d.; Adams, 2020; Amarasiri et al., 2022; Mtetwa et al., 2021; Rumky et al., 2022; Wong et al., 2015). Furthermore, none of these PCR methods were tested to our knowledge for their applicability in wastewater samples except for one study investigating a set of 10 ARG's related to tuberculosis treatment (*katG, rpoB, embB, pncA, rrs, gyrA, gyrB, atpE, ethR, eis*) using samples collected from WRRF in South Africa (Mtetwa et al., 2021).

The present study focuses on the standardization for the development and the validation of key AMR target ddPCR-based detection methods for wastewater surveillance. Therefore, in the context of the Belgian national action plan to fight AMR (NAP-AMR), we have developed a strategy for validation of ddPCR as a tool for AMR wastewater surveillance. Based on their global significance, we selected seven ARG's conferring resistance to extended spectrum beta-lactams ($bla_{CTX\text{-}M}$), carbapenems ($bla_{KPC\text{-}2/3}$) (Rodríguez et al., 2021), tetracyclines (*tet(M)*), erythromycin (*erm(B)*) (Heß et al., 2019), vancomycin (*vanA*) (Pärnänen et al., 2019), sulfonamides (*sul2*) (Adekanmbi et al., 2020), and aminoglycosides (*aac(3)V-IV*) (Obayiuwana & Ibekwe, 2020), as well as the class I integron (*intl1*) as an indicator of horizontal gene transfer (Alexander et al., 2020). Then, if available, we collected probe-based qPCR methods targeting these ARG's published in the scientific literature (Czekalski et al., 2014; Delannoy et al., 2022; McConnell et al., 2018; Schmidt et al., 2015; Volkmann et al., 2007). In addition, we constructed in-house inclusivity and exclusivity sequence databases, containing a total of 1.705.209 sequences, for in silico evaluation of these methods. Based on the results of this evaluation, the existing primers and probes were either kept or adapted for optimized PCR amplification. Moreover, if no convenient PCR method was available in scientific literature, new primers and probes were fully designed. Subsequently, when all the final primers and the probes were defined, four duplex ddPCR methods were designed. Their performances in terms of specificity, related to the absence of false-positive and false-negative

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results, and sensitivity, related to a limit of detection below 25 copies of the targets, were then assessed in order to comply with international standard performance criteria requirements (European Commission Joint Research Centre, 2023; Marchesi et al., 2015). Regarding the specificity evaluation, an in-house microbial collection of 36 bacterial strains fully characterized for ARG by whole-genome sequencing (WGS) and belonging to species previously reported as being frequently observed in wastewater was constructed. Finally, the applicability of the proposed ddPCR methods was tested on an in-house wastewater collection built with

TABLE 1 Sequences of primers and probes used for the four duplex digital droplet PCR (ddPCR) methods. In the first column, the assays are grouped together based on their respective duplex configurations. The second column specifies the targets for each assay. The third column provides the oligonucleotide sequences for the primers and probes. The nucleotides adapted in this study as well as the newly designed oligonucleotide sequences are highlighted in bold. The fourth column displays the amplicon size resulting from each assay. Lastly, the fifth column contains references to the original methods from which the assays were adapted or designed. For detailed information regarding the amplicon sequences, please refer to Data S1.

ddPCR methods	Targets	Oligonucleotides sequences (5′ → 3′)		Amplicon sizes	Original method references
Duplex-1	***bla*$_{CTX\text{-}M}$**	Forward	ACCAA**Y**GATAT**Y**GCGGT**K**AT	101 bp	Adapted from (Obayiuwana & Ibekwe, 2020)
		Probe	FAM-TCGTGCGCCGCTG-MGB-Eclipse		(Obayiuwana & Ibekwe, 2020)
		Reverse	ACATCGCG**R**CGGC**KY**TCT		Adapted from (Obayiuwana & Ibekwe, 2020)
	tet(M)	Forward	GGTTTCTCTTGGATACTTAAATCAATCR	88 bp	(Alexander et al., 2020)
		Probe	HEX-ATGCAGTTATGGARGGGATACGCTATGGY-3IABkFQ		
		Reverse	CCAACCATAYAATCCTTGTTCRC		
Duplex-2	***bla*$_{KPC\text{-}2/3}$**	Forward	AGCGGCAGCAGTTTGTTGAT	178 bp	(McConnell et al., 2018)
		Probe	FAM-CAGTCGGAGACAAAACCGGAACCTGC-3IABkFQ		
		Reverse	ACGGCCAACACAATAGGT		
	erm(B)	Forward	GGATTCTACAAGCGTACCTTGGA	90 bp	(Czekalski et al., 2014)
		Probe	HEX-CACTAGGGTTGCTCTTGCACACTCAAGTC-3IABkFQ		
		Reverse	TGGCAGCTTAAGCAATTGCT		
Duplex-3	***vanA***	Forward	CTGTGAGGTCGGTTGTGCG	65 bp	(Delannoy et al., 2022)
		Probe	FAM-CA**R**CTAACGCGGCACTGTTTCCCAAT-3IABkFQ		Adapted from (Delannoy et al., 2022)
		Reverse	TTTGGTCCACCTCGCCA		(Delannoy et al., 2022)
	sul2	Forward	GATATTCGCGGTTTTCCAGA	141 bp	(Czekalski et al., 2014)
		Probe	HEX-AAGACGGGCAGGCAGATCGG-3IABkFQ		
		Reverse	CGCAATGTGATCCATGATGT		
Duplex-4	***aac(3)-IV***	Forward	**CTCTGATCCATTGCCCCTG**	82 bp	This study
		Probe	FAM-**CACCTCACTCGCCTGCAAGCC**-3IABkFQ		
		Reverse	**GAGAAGTACCTGCCCATCG**		
	intl1	Forward	GCCTTGATGTTACCCGAGAG	196 bp	(McConnell et al., 2018)
		Probe	HEX-CGACGCCCTTGAGCGGAAGTATC-3IABkFQ		
		Reverse	GATCGGTCGAATGCGTGT		

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wastewater samples collected in two treatment plants in Brussels, Belgium. This study represents therefore a step forward in the wastewater community in order to reinforce the standardization methodology being essential for the global AMR surveillance requested by the EU Wastewater Treatment Directive proposal.

MATERIALS AND METHODS

PCR design and evaluation

Eight targets, including seven ARGs, *bla*$_{CTX-M}$, *bla*$_{KPC-2/3}$, *tet(M), erm(B), vanA, sul2, and aac(3)-IV*, and one indicator of (multi-)antibiotic resistance and horizontal gene transfer, *intl1*, were selected based on regional prevalence, expert advice, and clinical and epidemiological relevance (Adekanmbi et al., 2020; Alexander et al., 2020; Heß et al., 2019; Obayiuwana & Ibekwe, 2020; Pärnänen et al., 2019).

The first phase included a literature review for available PCR assays (Czekalski et al., 2014; Delannoy et al., 2022; McConnell et al., 2018; Schmidt et al., 2015; Volkmann et al., 2007) (Table 1, Data S1). Only probe-based assays, for an accurate identification, with amplicon lengths below 200 base pairs were considered for further evaluation. Then, *in silico* evaluations of the existing primers and probes were performed against two in house sequence databases, that is, the inclusivity and exclusivity sequence databases (see Sections 2.1.1 and 2.1.2). For the *aac(3)-IV* target, as no PCR method was published in the scientific literature, the primers and probes were designed within this study.

Inclusivity sequence database

A comprehensive sequence database encompassing the eight selected key AMR targets was established to facilitate the *in silico* inclusivity evaluation. The dataset was constructed by extracting ARG sequences from online available MicroBIGG-E ARG's sourced from the National Database of Antibiotic Resistant Organisms (NDARO) (Feldgarden et al., 2021) through NCBI. The targeted ARG sequences were acquired in tabular form and subsequently downloaded on August 23, 2023. To obtain the respective ARG sequences, an in-house R-script was developed. As the FASTA sequences were often corresponding to full bacterial genomes, this script enabled the retrieval and subsequent trimming of the downloaded bacterial FASTA sequences including the ARG's, retaining only the pertinent ARG sequence information. For the *intI1* target, the coding sequences and corresponding metadata were sourced from NCBI. An in-house Python script was devised to generate the intI1 dataset. All data were downloaded from NCBI on August 23, 2023. A total of 606,300 sequences were collected for the in-house inclusivity sequence database (Data S2).

Exclusivity sequence database

The exclusivity sequence database contains a diverse spectrum of organisms including animals, fungi, bacteria, viruses, plants, and protozoa. The species selection was done based on the literature (Chahal et al., 2016; Gerardi & Zimmerman, 2004; Mara & Horan, 2003; Olaolu, 2014) and metagenomics data from in-house sequenced wastewater samples (unpublished data). The latter was subjected to analysis with Kraken2 (Wood et al., 2019) and Krona (Ondov et al., 2011) tools within the Sciensano Galaxy framework. To ascertain accession numbers pertinent to the chosen organisms, the taxonomy browser of NCBI was consulted. The subsequent step encompassed the acquisition of metadata and corresponding FASTA sequences through an in-house developed R-script. Within this dataset, only European occurrences of animals and plants were retained. To reduce dataset dimensions for the other species, the identity tool (Girgis et al., 2021) was used with a threshold of 0.9, effectuating a reduction in the number of sequences. All data were downloaded from NCBI on March 29, 2023. A total of 1,098,909 sequences were collected for the in-house exclusivity sequence database (Data S2).

In silico assessment

The PCR assays were first evaluated against their corresponding inclusivity datasets using a custom in-house developed tool, named SCREENED, which is publicly available at https://galaxy.sciensano.be (Table 1). The SCREENED tool used in this study employed specific criteria for determining assay reliability. For instance, if no mismatches were detected within the first five nucleotides of the primers' 3′ end, and if the total number of mismatches did not exceed 10% of the oligonucleotide's length, the script considered that a positive signal would likely be generated (Gand et al., 2021). The inclusivity of each primer or probe was assessed, with a threshold set to deem inclusivity as low if it fell below 80%. Any primers or probes identified with low inclusivity were subsequently modified for enhanced performance (Table 1). Given the absence of PCR assays targeting the aac(3)-IV gene in existing literature, new assays were manually designed. This was achieved using the aac(3)-

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IV inclusivity database. All these sequences were aligned using mafft and MEGA11. On this basis, conservative regions were identified and selected to subsequently manually design primers and probe, checking in parallel the oligonucleotide parameters with IDT OligoAnalyzer tool (Table 1).

To select optimal duplex assays, the IDT OligoAnalyzer tool (Owczarzy et al., 2008) was used to evaluate heterodimer interactions with the focus on the minimal delta G value, resulting in the selection of four duplex assays: *bla*$_{CTX-M}$—*tet(M), bla*$_{KPC-2/3}$—*erm(B), vanA—sul2*, and *aac(3)-IV—intI1*. To validate their accuracy, the SCREENED tool was employed to test their performance against the exclusivity sequence database. Since there are bacteria included in the database, it is possible that positive results are revealed. These positive results were corroborated by using an external tool, being Resfinder4.1.11 (using kma). The bacterial strains within the exclusivity sequence database were subjected to analysis using Resfinder 4.1.11 with a minimum coverage of 60% and a threshold for identity of 90% (Bortolaia et al., 2020).

Once the ddPCR methods were developed in silico, we assessed the performances of the four duplex assays in terms of specificity, sensitivity, and applicability. The method specificity assessment is performed using positive and negative materials to verify if "a PCR module produce only amplification products with the target sequence for which the PCR module was developed". The method sensitivity assessment is performed using serial dilutions of the PCR target(s) to determine the limit of detection, being "the lowest amount of analyte in a sample, which can be reliably detected". The method applicability assessment is performed using "sample materials to which the PCR module can be applied in order to demonstrate the fitness for purpose of the PCR module with respect to the scope of application" (European Commission Joint Research Centre, 2023; Marchesi et al., 2015).

Experimental specificity assessment

On the one hand, microbial species, which are most commonly found in wastewater, were identified based on literature review and include *Escherichia coli*, *Salmonella* spp., *Shigella* spp., *Campylobacter* spp., *Trichoderma* spp., *Aspergillus* spp., and *Candida* spp. (Chahal et al., 2016; Gerardi & Zimmerman, 2004; Mara & Horan, 2003; Olaolu, 2014). On the other hand, bacterial species commonly known in scientific literature to carry the eight AMR targets of interest were identified and include *Enterobacteriaceae* and *Pseudomonadaceae* for *erm(B)* (Batista et al., 2023), *Klebsiella* spp. for *sul2* and *bla*$_{KPC-2/3}$ (Baba et al., 2022; Obayiuwana & Ibekwe, 2020), *E. coli* for *tet(M)* and *bla*$_{CTX-M}$ (Alexander et al., 2020; Baba et al., 2022; Batista et al., 2023; Liguori et al., 2022; Rodríguez et al., 2021; Shin et al., 2021), *E. coli* and *Klebsiella* spp. (Baba et al., 2022; Obayiuwana & Ibekwe, 2020; Shin et al., 2021) for *aac(3)-IV*, and *Enterococcus* spp. for *vanA* (Alexander et al., 2020). All these organisms were listed either because they were reported as being frequently observed in wastewater and/or they were carrying at least one of the eight AMR targets of interest. Based on this assessment, a selection was done to experimentally test and evaluate the specificity of the developed methods. The main objective of this selection was to efficiently cover all AMR targets of interest by testing a rational and minimal number of bacterial species, if possible, previously observed in wastewater (such as *E. coli* and *Salmonella* spp.). More precisely, an in-house microbial collection was built, composed of DNA from 36 bacterial strains. It includes a diverse spectrum of bacterial strains like one *Campylobacter jejuni*, two *Enterococcus faecium*, 18 *E. coli*, two *Salmonella enterica* serovar Typhimurium variant Monophasic, one *S. enterica* serovar Rissen, two *S. enterica* serovar Typhimurium, two *Salmonella infantis*, one *Salmonella Typhi*, one *Shigella boydii*, and six *Shigella sonnei* (Table 2, Data S3). The WGS data from these 36 bacterial strains were analyzed using Resfinder 4.1.11 to determine whether or not they contained the eight AMR targets of interest, in order to determine false and negative materials for the tested ddPCR methods. DNA from these 36 bacterial strains was extracted using the NucleoSpin® Food Genomic DNA Extraction Kit (Macherey-Nagel, USA), the MagCore Genomic DNA Bacterial Kit (RBCBioscience, USA), the GenElute Bacterial Genomic DNA Extraction Kit (Sigma-Aldrich, USA), or the Maxwell RSC Cultured Cells DNA Kit (Promega, USA). In addition, DNA from one fungus, a human, and one plant was collected as negative material for the ddPCR method's specificity evaluation (Table 2, Data S3). DNA from *Trichoderma reesei* (strain collection number IHEM 5648, BCCM collection, Belgium) was extracted using the NucleoSpin® Food Genomic DNA Extraction Kit (Macherey-Nagel, USA). Commercial *Homo sapiens* DNA was used (collection number G152A, Promega, Belgium). DNA from *Oryza sativa* (collection number AOCS 0306-D2, American oil chemists' society, USA) was extracted as previously described (Broeders et al., 2015).

For all these DNA materials, DNA concentrations were measured by fluorometry with the Qubit 3.0 fluorometer (ThermoFisher, Belgium), and 0.01 ng in duplicate was tested for each ddPCR method. For each ddPCR method, a synthetic control plasmid artificially

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TABLE 2 Experimental specificity assessment using the in-house microbial DNA collection and synthetic control plasmids.

			ddPCR methods							
			Duplex-1		Duplex-2		Duplex-3		Duplex-4	
Kingdom	**Species**	**Collection numbers**	***bla_{CTX-M}***	***tet(M)***	***$bla_{KPC-2/3}$***	***erm(B)***	***vanA***	***sul2***	***aac(3)-IV***	***intI1***
Animalia	*H. sapiens*	G152A	–	–	–	–	–	–	–	–
Plantae	*O. sativa*	AOCS 0306-D2	–	–	–	–	–	–	–	–
Fungi	*T. reesei*	IHEM 5648	–	–	–	–	–	–	–	–
Bacteria	*Campylobacter jejuni*	S23FP03039	–	+	–	+	–	–	–	–
	Enterococcus faecium	LMG 16171, LMG 16474	–	+	–	+	+	–	–	–
	E. coli	EC KPC-2 EURL	–	–	+	–	–	–	–	–
		EH2130	–	–	–	+	–	–	–	+
		EH2303, S21FP00929, S21FP01851, S21FP02278	+	–	–	–	–	–	–	+
		S21FP01344, S21FP02612	+	–	–	–	–	+	–	+
		S21FP01478, S21FP01596	+	–	–	+	–	+	–	+
		S21FP01831, S21FP04634	+	–	–	–	–	+	–	–
		S21FP01857, TIAC3619	–	–	–	–	–	+	–	+
		S21FP02610, TIAC2906	–	+	–	+	–	+	–	+
		TIAC3832	–	–	–	–	–	–	–	+
		TIAC5245	–	+	–	–	–	+	–	+
	Salmonella enterica serovar typhimurium variant monophasic	S16BD07964	+	–	–	–	–	+	–	–
		S17BD04785	+	–	–	–	–	–	–	+
	S. enterica serovar Rissen	S16BD01842	+	–	–	–	–	+	–	–
	S. enterica serovar typhimurium	S16BD06461	+	–	–	–	–	+	–	+
		S18BD06686	+	–	–	–	–	–	+	+
	Streptococcus infantis	S17BD06931	+	–	–	–	–	+	–	+
		S18BD02037	+	–	–	–	–	–	+	+
	S. Typhi	S17BD03474	+	–	–	–	–	+	–	+
	Shigella boydii	S18BD07286	+	–	–	+	–	+	–	–
	Shigella sonnei	S13BD04362	+	–	–	–	–	–	–	–
		S15BD06969	+	–	–	–	–	+	–	+
		S15BD09162, S17BD04542, S18BD05550	+	–	–	–	–	+	–	–
		S18BD03411	+	–	–	–	–	–	–	+
Synthetic control plasmids		*bla_{CTX-M}*	+	–	–	–	–	–	–	–
		tet(M)	–	+	–	–	–	–	–	–
		$bla_{KPC-2/3}$	–	–	+	–	–	–	–	–

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TABLE 2 (Continued)

			ddPCR methods							
			Duplex-1		Duplex-2		Duplex-3		Duplex-4	
Kingdom	Species	Collection numbers	*bla*$_{CTX-M}$	*tet (M)*	*bla*$_{KPC-2/3}$	*erm (B)*	*vanA*	*sul2*	*aac(3)-IV*	*intI1*
		erm(B)	–	–	–	+	–	–	–	–
		vanA	–	–	–	–	+	–	–	–
		sul2	–	–	–	–	–	+	–	–
		aac(3)-IV	–	–	–	–	–	–	+	–
		intI1	–	–	–	–	–	–	–	+

The absence and presence of amplification are symbolized by "-"and "+," respectively. The digital droplet PCR (ddPCR) assays with less than three positive droplets were considered as negative. For each result, the experiment was performed in duplicate (Data S3). The gray cases correspond to the target genes detected by whole-genome sequencing (WGS) for each strain.

synthetized to carry a single copy of the generated corresponding PCR amplicon was designed and purchased (Genecust, France) (Table 2, Data S1). These eight synthetic control plasmids were individually tested at 10,000 copies in duplicate for each ddPCR method.

Experimental sensitivity assessment

The sensitivity of each ddPCR method was evaluated using serial dilutions of the corresponding synthetic control plasmid that have been artificially synthetized to carry a single copy of the generated PCR amplicon (Genecust, France) (Table 3A, Data 1). For each of these synthetic control plasmids, six dilution points were prepared, that is, 20, 10, 5, 2, 1, and 0.1 estimated target copies. Each dilution point was tested in 12 replicates. The limit of detection ($LOD_{95\%}$), defined as the number of target copies required to ensure a 95% probability of detection (POD), was calculated using the QuoData web application (Data S4) (Uhlig et al., 2015).

For multiplex methods, the asymmetric sensitivity also needs to be evaluated, that is, one target is tested at low concentration in the presence of high concentrations of the other(s) target(s) and vice versa (Table 3B) (European Commission, n.d.). The asymmetric sensitivity of our duplex methods was tested using synthetic control plasmid mixtures. For each duplex method, two synthetic control plasmid mixtures were prepared: (i) one containing 20,000 estimated target copies of Target 1 from the duplex method and 20 estimated target copies of Target 2 from the duplex method and (ii) one containing 20 estimated target copies of Target 1 from the duplex method and 20,000 estimated target copies of Target 2 from the duplex method. Each synthetic control plasmid mixture was tested in 12 replicates.

Applicability assessment

First, we built an in-house wastewater sample collection from two Belgian WRRF (Bruxelles-Nord/Brussel-Noord (WRRF-A); Bruxelles-Sud/Brussel-Zuid (WRRF-B)). Samples are collected on Mondays or Wednesday by autosamplers (24-h composite) at the influent of the WRRFs. For WRRF-A, the sampler uses a volume proportional mode (100 mL every 1200 m^3 reaching the WRRF). For WRRF-B, the sampler also uses a volume proportional mode (50 mL every 1000 m^3 reaching the WRRF) (Broeders et al., 2015). These wastewater samples were collected in the frame of the National Surveillance each week for 13 months, going from September 2022 to September 2023. Once collected, the samples were transported the same day at 4°C and subsequently either directly stored at −20°C or directly extracted for DNA/RNA to be then stored at −80°C. From this large wastewater sample collection, a total of 14 samples, covering one sample for each 2 months per WRRF, were selected in this study for the applicability assessment of the proposed ddPCR methods (Figure 1, Data S5).

For each developed ddPCR method, DNA extracted from the 14 wastewater samples were used. These samples were collected using 24 h time-proportional autosamplers; 50 mL of wastewater was concentrated by ultrafiltration Centricon Plus-70 Centrifugal filters (100 kDa). The validated nucleic acid extraction protocol, using Maxwell® RSC PureFood GMO and Authentication Kit (Promega, USA), was used (Janssens et al., 2022). Per ddPCR reaction, 1.5 μL of DNA (resulting from a dilution 8 of the original DNA extract) were tested in duplicate. Positive controls, including the eight synthetic control plasmids (Data S1), and no template control (NTC) were included in each ddPCR assay.

The R-script used to construct Figure 1 is available in Data S6.

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TABLE 3 Experimental sensitivity assessment. The absence and presence of amplification are symbolized by "-" and "+," respectively. The digital droplet PCR (ddPCR) assays with less than three positive droplets were considered as negative.

(A)

Estimated target copies	**Duplex-1**		**Duplex-2**		**Duplex-3**		**Duplex-4**	
	***bla*$_{CTX-M}$**	***tet(M)***	***bla*$_{KPC-2/3}$**	***erm(B)***	***vanA***	***sul2***	***aac(3)-IV***	***intl1***
20	+ (12/12) (18.1 ± 8.9 cp)	+ (12/12) (18.4 ± 5.6 cp)	+ (12/12) (19.1 ± 6.4 cp)	+ (12/12) (23.2 ± 6.0 cp)	+ (12/12) (15.4 ± 6.3 cp)	+ (12/12) (11.9 ± 3.6 cp)	+ (12/12) (12.7 ± 4.2 cp)	+ (12/12) (16.7 ± 3.6 cp)
10	+ (11/12) (9.6 ± 6.2 cp)	+ (12/12) (9.4 ± 4.8 cp)	+ (12/12) (8.3 ± 4.0 cp)	+ (12/12) (10.8 ± 3.9 cp)	+ (12/12) (7.8 ± 5.2 cp)	+ (11/12) (6.9 ± 3.0 cp)	+ (12/12) (4.1 ± 1.9 cp)	+ (12/12) (7.8 ± 2.6 cp)
5	+ (8/12) (3.0 ± 2.4 cp)	+ (11/12) (6.4 ± 3.5 cp)	+ (6/12) (2.5 ± 2.6 cp)	+ (8/12) (6.1 ± 6.2 cp)	+ (11/12) (5.0 ± 3.6 cp)	+ (7/12) (4.0 ± 3.7 cp)	+ (11/12) (2.8 ± 2.0 cp)	+ (7/12) (2.5 ± 2.3 cp)
2	+ (3/12) (1.4 ± 2.7 cp)	+ (5/12) (1.6 ± 1.9 cp)	+ (3/12) (1.2 ± 2.1 cp)	+ (3/12) (1.2 ± 2.1 cp)	+ (8/12) (1.0 ± 2.3 cp)	+ (4/12) (0.3 ± 0.5 cp)	+ (9/12) (1.1 ± 0.7 cp)	+ (3/12) (0.9 ± 1.6 cp)
1	− (0/12)	+ (1/12) (0.3 ± 1.1 cp)	+ (1/12) (0.3 ± 1.1 cp)	+ (1/12) (0.4 ± 1.3 cp)	− (0/12)	− (0/12)	− (0/12)	+ (1/12) (0.3 ± 1.0 cp)
0.1	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)
0	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)	− (0/12)
LOD$_{95\%}$ (copies)	**16**	**9**	**15**	**13**	**8**	**16**	**7**	**14**

(B)

Duplex methods	**Synthetic control plasmid mixtures**	**Target 1**	**Target 2**
Duplex-1	bla$_{CTX-M}$ 20000 cp + tet(M) 20 cp	+ (24138.3 ± 319.6 cp)	+ (21.7 ± 5.3 cp)
bla$_{CTX-M}$ - tet(M)	bla$_{CTX-M}$ 20 cp + tet(M) 20,000 cp	+ (18.8 ± 7.5 cp)	+ (24346.7 ± 346.1 cp)
Duplex-2	bla$_{KPC-2/3}$ 20,000 cp + erm(B) 20 cp	+ (20926.7 ± 466.4 cp)	+ (24.9 ± 6.4 cp)
bla$_{KPC-2/3}$ - erm(B)	bla$_{KPC-2/3}$ 20 cp + erm(B) 20,000 cp	+ (15.3 ± 5.8 cp)	+ (21600.0 ± 313.1 cp)
Duplex-3 *vanA - sul2*	vanA 20,000 cp + sul2 20 cp	+ (21846.7 ± 5.8 cp)	+ (15.0 ± 3.5 cp)
	vanA 20 cp + sul2 20,000 cp	+ (21.0 ± 5.8 cp)	+ (21670.0 ± 407.7 cp)

(Continues)

TABLE 3 (Continued)

(B)			
Duplex methods	**Synthetic control plasmid mixtures**	**Target 1**	**Target 2**
Duplex-4	aac(3)-IV 20000 cp + intl1 20 cp	+ (23803.3 ± 491.3 cp)	+ (23.8 ± 2.8 cp)
aac(3)-IV - intl1	aac(3)-IV 20 cp + intl1 20,000 cp	+ (23.6 ± 7.4 cp)	+ (22600.03 ± 421.0 cp)

Note: (A) Symmetric sensitivity. For each estimated target copy, 12 replicates were tested. The number of positive replicates and the mean values of the measured copies (cp) are indicated. The calculated $LOD_{95\%}$ is also shown (Data S4).
Note: (B) Asymmetric sensitivity. For each synthetic control plasmid mixture, 12 replicates were tested, and the mean values of the measured copies (cp) are indicated.

FIGURE 1 Applicability assessment of the developed digital droplet PCR (ddPCR) methods. Boxplot graph showing the ddPCR results for 14 wastewater samples expressed in log copies/mL wastewater. The experiment was performed in duplicate on each wastewater sample. The ddPCR assays with less than three positive droplets were considered as negative. See Data S5 for the ddPCR values.

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ddPCR assays

Each duplex ddPCR reaction was performed in a volume of 20 μL, containing 2× ddPCR Supermix for probes (no dUTP) (Bio-Rad, Belgium), 900 nM of each primer (IDT, Belgium), 250 nM of each probe (IDT, Belgium), and 5 μL of DNA. Sequences of primers and probes used for the four duplex ddPCR methods are listed in Table 1 (Data S1). According to the manufacturer's instructions, 20 μL of the reaction mix and 70 μL of droplet generation oil for probes (Bio-Rad, Belgium) were loaded into a DG8™ cartridge (Bio-Rad, Belgium) and then placed in a QX200™ droplet generator (Bio-Rad, Belgium). A PCR amplification was performed on 40 μL of the generated droplets using a T100™ thermal cycler (Bio-Rad, Belgium) with the following program: one cycle at 25°C for 3 min, one cycle at 95°C for 10 min (Taq polymerase activation), 40 cycles at 94°C for 30 s (denaturation), 58.5°C for 1 min (annealing-extension), and one cycle at 98°C for 10 min (Taq polymerase inactivation).

Using the QX200™ droplet reader (Bio-Rad, Belgium), the raw data were acquired and analyzed with QuantaSoft software version 1.7.4.0917 (Bio-Rad, Belgium). The threshold was manually set between the cloud of positive and negative droplets. The ddPCR assays with less than three positive droplets were considered as negative (Capo et al., 2021; Drandi et al., 2020). For each assay, a NTC was included.

RESULTS AND DISCUSSION

In silico assessment

Inclusivity

The *in silico* inclusivity evaluation was carried out by assessing primers and probes against their corresponding inclusivity databases tailored for each AMR target. The *in silico* evaluation of the PCR assays revealed high inclusivity for the targeted regions (Data 2A). The substantial size of the inclusivity database (606,300 sequences) underscores the comprehensiveness of the inclusivity assessment, enhancing the confidence in the reliability of the assays. For each AMR target, no false positive was observed since no hit was resulting with sequences belonging to the other AMR target datasets. Moreover, for each AMR target tested against its corresponding dataset, a high inclusivity result was observed. For example, for the $bla_{CTX\text{-}M}$, *tet(M)*, $bla_{\text{KPC-2/3}}$, *erm(B)*, *vanA*, *sul2,* and *aac(3)-IV*, a very high inclusivity result was obtained, ranging from 97.31% to 99.89%. Although the inclusivity of integron 1 (*intl1*) is still high (88.22%), the presence of mismatches warrants attention. These mismatches are likely attributed to discrepancies within the database. This is likely due to the fact that in contrast to the AMR, there was no predefined NCBI table available to facilitate the selection of appropriate sequences, which could have contributed to database miscategorization, resulting in mismatches.

Exclusivity

An extensive assessment of the exclusivity of the primers and probes was conducted across a diverse range of organisms, including eukaryotes, protozoa, viruses, and bacteria. The *in silico* results indicated no positive results for all organisms, except for some bacteria. More precisely, for all AMR targets, except *vanA*, a low positive result for exclusivity was observed, going from 0.01% to 1.55%. This observation aligns with the expectations because some bacteria have ARG's and integrons (Data 2B). These findings were confirmed using Resfinder 4.1.11 as an independent verification tool using kma. In the majority of the cases, the obtained results are successfully corroborated by Resfinder 4.1.11 (Data 2B). The lower concordance observed for $bla_{CTX\text{-}M}$ is likely caused by closely related ARG (e.g., other *bla* genes).

Experimental specificity assessment

The specificity of the developed ddPCR methods was experimentally assessed by testing DNA materials from our in-house microbial collection, including 36 bacterial strains characterized by WGS and belonging to species previously reported as being frequently observed in wastewater (Table 2, Data S3). These strains belong to *E. coli, Salmonella* spp.*, Shigella* spp., *Enterococcus* spp., and *Campylobacter* spp. (Chahal et al., 2016; Gerardi & Zimmerman, 2005; Mara & Horan, 2003; Olaolu, 2014). In addition, one fungal, one human, and one plant DNA were used as negative controls. Moreover, eight synthetic control plasmids were used as positive controls for the corresponding ddPCR method and as negative controls for the other ddPCR methods. As expected, for each ddPCR method, a negative signal was observed for all the negative controls, while the positive controls showed an amplification for the corresponding target (Table 2). Regarding the 36 bacterial strains, positive and negative signals were observed in accordance with the ARG information extracted from in silico analysis and the WGS data analysis (Table 2). Given that only expected target sequences were detected by the expected ddPCR method (100% of true-positive and true-negative signals) and that

no false-positive and false-negative results (0% of false-positive and false-negative signals) were observed, the developed ddPCR methods were assessed as specific.

Experimental sensitivity assessment

For the symmetric sensitivity assessment of the developed duplex methods, serial dilutions of each synthetic control plasmid, ranging from 20 to 0.1 estimated target copies, were used (Table 3A). A positive signal was observed for all 12 replicates as low as 20 estimated target copies for the $bla_{CTX\text{-}M}$ and *sul(2)* methods and as low as 10 estimated target copies for the *tet(M),* $bla_{KPC\text{-}2/3}$*, erm(B), vanA, aac(3)-IV,* and *intl1* methods. As expected, no positive signal was observed at 0 estimated target copy (Table 3A). Based on all positive and negative signals observed for the 12 replicates at each serial dilution point tested, the $LOD_{95\%}$ of each developed ddPCR method was calculated and determined to be between seven and 16 estimated target copies, depending on the target (Table 3A, Data S4). Given the $LOD_{95\%}$ lower than 25 estimated target copies, the developed ddPCR methods are assessed as sensitive and their performance comply with the "Minimum Performance Requirements for Analytical Methods of GMO Testing" of the European Network of GMO Reference Laboratories (European Commission Joint Research Centre, 2023; Marchesi et al., 2015).

For the asymmetric sensitivity, each duplex method was tested using synthetic control plasmid mixtures composed either of 20,000 estimated target copies of Target 1 and 20 estimated target copies of Target 2, or inversely (Table 3B). For each duplex method and for each synthetic control plasmid mixture, a positive signal was observed for all 12 replicates, and the measured target copy number was close to the expected value (20,000 or 20) (Table 3B). These results show that the duplex methods are not impacted when one target is highly abundant compared to the other.

Applicability assessment

The applicability of the developed ddPCR methods was experimentally assessed by testing 14 DNA extracts from wastewater samples (Figure 1, Data S5). The wastewater samples show both positive and negative signals for the tested targets. The $bla_{CTX\text{-}M}$, $bla_{KPC\text{-}2/3}$, *vanA*, and *aac(3)-IV* targets present low ddPCR signals between 0 and 838 copies/mL wastewater. And the *tet(M), erm(B), sul2*, and *intl1* targets present high ddPCR signals ranging from 2341 to 115,670 copies/mL wastewater. The abundance of AMR targets, including the eight ones selected in this study, was previously investigated in wastewater by several studies using mainly qPCR technology. The abundance of these eight AMR targets has usually been categorized in three groups: high abundance (*intl1* (Alexander et al., 2020; Liguori et al., 2022), *erm(B)*, *sul2* (Rodríguez et al., 2021), and *tet(M)* (Alexander et al., 2020; Pärnänen et al., 2019)); medium abundance ($bla_{CTX\text{-}M}$ (Alexander et al., 2020; Batista et al., 2023; Liguori et al., 2022; Rodríguez et al., 2021; Shin et al., 2021)) and low abundance (*aac(3)-IV* (Obayiuwana & Ibekwe, 2020), *vanA* (Alexander et al., 2020), and $bla_{KPC\text{-}2/3}$ (Ferreira et al., 2023)). In addition, $bla_{CTX\text{-}M}$ was also reported by one study as found in low abundancy (Liguori et al., 2022). The results obtained in this study related to the abundancy of the eight investigated AMR targets in wastewater are consistent with previously reported observations (Alexander et al., 2020; Ferreira et al., 2023; Liguori et al., 2022; Obayiuwana & Ibekwe, 2020; Pärnänen et al., 2019).

CONCLUSION

The potential of wastewater surveillance as a tool to monitor ARG's is increasingly recognized. Moreover, with a directive proposal at the EU level urging member states to monitor antibiotic resistance in WRRF, the need for a reliable and harmonized approach to AMR monitoring in wastewater becomes essential for meaningful data comparison between countries. In this context, the use of a similar methodology, including a harmonized workflow for detection method validation, is essential.

This study presents a strategy to address the needs related to the standardization in the development and validation of qPCR and ddPCR methods for global AMR surveillance in wastewater. As a case study, four duplex ddPCR methods focusing on seven ARG's, $bla_{CTX\text{-}M}$, $bla_{KPC\text{-}2/3}$*, tet(M), erm(B), vanA, sul2*, and *aac(3)-IV*, and one AMR indicator, *intl1*, were developed and validated. The proposed strategy, designed to be modular for potential inclusion of additional key AMR targets, encompasses several key steps.

First, an *in silico* specificity assessment of the ddPCR methods was performed, supported by the construction of an in-house sequence database. This database, being essential, not only aids in the *in silico* specificity assessment of the ddPCR methods, allowing oligonucleotide adaptations of existing methods and new oligonucleotide designs, but also forms a foundation for a shared resource among laboratories engaged in EU AMR surveillance, fostering collaboration and harmonization by sharing as much as possible sequence databases with the EU AMR surveillance community. Moreover, this *in silico*

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specificity evaluation offers the advantage of testing a very large number of sequences, whereas, for the experimental specificity assessment, only a limited number of materials can realistically be considered. In this study, a substantial size of sequences for the *in silico* specificity assessment was used, including 606,300 sequences for inclusivity and 1,098,909 sequences for exclusivity, enhancing the confidence in the reliability of the assays.

Second, experimental specificity and sensitivity assessments of the ddPCR methods were conducted. Currently, no standard guidance document specific for the development and validation of PCR-based methods to monitor AMR targets in wastewater exists. Therefore, the well-described and established standard guidance documents for PCR-based method validation in the context of GMO control were used (European Commission Joint Research Centre, 2023; Marchesi et al., 2015). The ddPCR method performance was assessed using an in-house constructed microbial DNA collection of 36 bacterial strains belonging to species reported as being commonly observed in wastewater. These strains were also characterized using WGS to determine whether they carried the selected AMR targets of interest. This type of microbial DNA collection is essential for method validation and can be also shared among involved laboratories to harmonize EU AMR surveillance. Regarding the method sensitivity, the detection limit was determined as below 20 copies of the targets. These ddPCR methods are consequently considered as being highly sensitive (European Commission Joint Research Centre, 2023; Marchesi et al., 2015). However, although the sensitivity evaluation is essential to generate methods that will then be used by different countries for global AMR surveillance in wastewater, the threshold and minimum required performance in terms of method sensitivity in this particular context needs to be discussed by the competent authorities and experts, in order to be in line with needs for an efficient and harmonized EU AMR surveillance in wastewater.

Finally, the validated ddPCR methods were tested for their applicability using several samples from an in-house wastewater collection from two Belgian WRRF, confirming their applicability as generating results consistent with scientific literature on abundance of ARG's.

Although further efforts are needed, this study represents a significant step forward towards a harmonized and reliable global AMR surveillance tool in wastewater at EU level, as requested by the new EU Wastewater Treatment Directive proposal. By providing a standardized approach to qPCR and ddPCR method development and validation, this strategy lays the groundwork for enhanced collaboration and data comparability among member states, ultimately strengthening efforts to combat AMR on a global scale. The proposed strategy will therefore be reinforced by sharing sequence databases between members of the wastewater community, as well as by using a common minimal set of positive and negative materials from shared microbial collections, such as through standardized wastewater mock-community samples. Moreover, the modular nature of this strategy allows for easy adaptation to incorporate new key AMR targets as needed, ensuring its relevance and applicability in evolving surveillance needs. Finally, the proposed strategy represents also a support in the establishment of appropriate guidance documents specific for the wastewater community.

AUTHOR CONTRIBUTIONS

Conceptualization: Pieter-Jan Ceyssens and Nancy Roosens. *Project administration*: All authors. *Data curation*: Andrea Gobbo, Marie-Alice Fraiture, Laura Van Poelvoorde, Hadrien Maloux, Veronik Hutse, and Nancy Roosens. *Methodology*: Andrea Gobbo, Marie-Alice Fraiture, Laura Van Poelvoorde, Sigrid C. J. De Keersmaecker, Cristina Garcia-Graells, Koenraad Van Hoorde, Bavo Verhaegen, Astrid Huwaert, Pieter-Jan Ceyssens, and Nancy Roosens. *Formal analysis*: Andrea Gobbo, Marie-Alice Fraiture, Laura Van Poelvoorde, Cristina Garcia-Graells, Hadrien Maloux, and Veronik Hutse. *Validation*: Andrea Gobbo, Marie-Alice Fraiture, Pieter-Jan Ceyssens, and Nancy Roosens. *Investigation*: Andrea Gobbo, Marie-Alice Fraiture, Laura Van Poelvoorde, Pieter-Jan Ceyssens, and Nancy Roosens. *Visualization*: Andrea Gobbo, Marie-Alice Fraiture, Laura Van Poelvoorde, and Nancy Roosens. *Writing—original draft preparation*: Andrea Gobbo, Marie-Alice Fraiture, and Laura Van Poelvoorde. *Writing—review and editing*: All authors. *Funding acquisition*: Pieter-Jan Ceyssens and Nancy Roosens. *Supervision*: Marie-Alice Fraiture and Nancy Roosens.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there are no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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