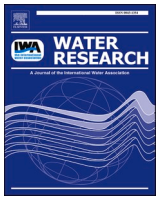
Water Research 287 (2025) 124379

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres



Tracking wild-type measles virus in wastewater using multiplex RT-dPCR, A novel tool for measles surveillance

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ARTICLE INFO

Keywords:
Wastewater surveillance
wastewater-based epidemiology
measles
digital PCR
public health

ABSTRACT

A multiplex digital RT-PCR (RT-dPCR) assay targeting three distinct regions of the measles virus genome was developed for wastewater surveillance. This method was applied to 40 mL samples collected at the inlets of two French wastewater treatment plants located in urban areas where clinical measles cases involving genotypes B3 and D8 had been reported. Detection was performed retrospectively on weekly samples collected between January and July 2024 as part of the routine national SARS CoV-2 wastewater surveillance network. Positive results were obtained in 27.3 % and 66.7 % of samples, with viral concentrations ranging between 5.8×10^2 gc/L – 4.6×10^3 gc/L and 1.8×10^3 gc/L – 2.2×10^4 gc/L, respectively. A complementary RT-dPCR, specifically targeting vaccine strain genomes, confirmed that detected signals originated from wild-type viruses and not from recent vaccination. These results confirm that the developed multiplex RT-dPCR assay can reliably detect measles virus in wastewater, even in areas with a low number of reported cases. Wastewater surveillance therefore represents a promising complementary tool for monitoring measles circulation at the community level and could support disease elimination efforts by detecting low-level residual circulation that may go undetected by clinical surveillance.

1. Introduction

Since 2022, there has been a resurgence of measles outbreaks worldwide, driven by several years of declining vaccination coverage and disrupted immunization schedules following the COVID-19 pandemic (WHO, 2020). This trend has affected the European region, with 127 350 cases of measles reported in 2024, twice as many as in 2023 and the highest annual total since 1997 (UNICEF and WHO/Europe, 2025). In 2024, 483 measles cases were reported in France, a fourfold increase compared to 2023 (Santé Publique France, 2025). Given the current epidemiological situation, and in line with the WHO Strategic Framework for Measles and Rubella Control 2021-2030, which aims to strengthen surveillance for rapid detection of outbreaks and timely response, it is essential to enhance surveillance for the rapid detection of measles virus circulation (Geneva: WHO, 2020).

Measles is an enveloped RNA virus that spreads through airborne transmission. The virus causes one of the most highly contagious diseases, with the basic reproduction number (R0) – representing the average number of secondary cases that would occur when an infectious agent is introduced into a susceptible population – generally estimated between 12 and 18 (Guerra et al., 2017; Moss and Griffin, 2006). Viral particles have been widely detected in several human biological fluid such as blood, saliva, and nasopharyngeal secretions from respiratory tract of infected individuals (Hübschen et al., 2022; Moss and Griffin, 2006), but are primarily excreted through urine (Chen and Bibby, 2023).

Wastewater-based epidemiology (WBE) has developed considerably

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https://doi.org/10.1016/j.watres.2025.124379
Received 13 June 2025; Received in revised form 31 July 2025; Accepted 8 August 2025
Available online 9 August 2025

in the wake of the COVID-19 pandemic and is considered a valuable tool to inform public health responses, especially when individual-level testing is not widely available. However, extending this surveillance to other infectious agents remains challenging (Bubba et al., 2023; National Academies of Sciences, Engineering, and Medicine, 2023; Shaw et al., 2023). Measles virus has been suggested on several occasions as a promising target for wastewater surveillance (WWS) (Chen and Bibby, 2023; Gentry et al., 2023; Toro et al., 2024; WHO, 2024). Indeed, such surveillance could support early detection objectives, improve understanding of viral load or activity within a population, and provide additional evidence to confirm the end of a circulation event. However, experience with detecting measles virus in wastewater is still limited (Benschop et al., 2017; Rector et al., 2025), and many studies have only been carried out on spiked wastewater samples due to the lack of measles virus circulation in the population to obtain field samples (Farkas et al., 2022; Hayes et al., 2023; Wu et al., 2024). Thus, evidence of detection *in natura* remains tenuous.

The aim of the proposed work was to develop, evaluate, and validate an analytical strategy based on the detection and quantification of the measles virus genome in raw urban wastewater using multiplex digital RT-PCR (RT-dPCR) methods. Viral quantification was performed using three sets of primers/probes targeting different parts of the genome, including the nucleocapsid gene, as recommended by the World Health Organization for measles monitoring. This gene is currently used for measles screening in clinical samples by the French National Reference Center (NRC) for Measles and accordingly served as reference system in this study. In addition, as measles is an integral part of vaccination programs through the measles, mumps, and rubella (MMR) vaccine, which is a live attenuated virus vaccine excreted in urine (Eckerle et al., 2013; Kaic et al., 2010; Rota et al., 1995), a genome screening system for vaccine products was assessed in parallel to distinguish between vaccine strains and wild-type viruses. The proposed analytical strategy was evaluated and validated using wastewater samples collected in two urban areas where measles cases had been reported.

2. Materials and methods

2.1. Wastewater samples collection and processing

2.1.1. Sampling location

Retrospective samples were collected from three wastewater treatment plants (WWTPs) followed within France's national wastewater surveillance network, SUM'EAU (Jourdain et al., 2025). Lyon Saint-Fons (Auvergne-Rhône-Alpes region, Rhône department) and Nîmes (Occitanie region, Gard department) were selected due recent measles circulation in these areas identified at the time of the study. Maxéville (Lorraine, Grand Est region, Meurthe-et-Moselle department) was chosen as a background control. These WWTPs serve catchment areas of approximately 600 000, 150 000, and 275 000 inhabitants, respectively.

2.1.2. Sample selection

Composite samples proportional to the incoming wastewater flow, were taken weekly from March 12th to July 9th 2024 (Lyon Saint-Fons), February 13th to July 10th 2024 (Nîmes), and bi-weekly from January 1st to July 1st 2024 (Maxéville). Those samples were obtained from SUM'EAU's laboratory network and transported at 5°C ± 3°C (Jourdain et al., 2025). N-NH_4 measurements were performed in accordance with the NF T90-015-1 standard. More information on sample characteristics can be found in Supplementary Table S1.

2.1.3. Sample concentration and nucleic acid extraction

Samples were processed using the Zymo Environ Water RNA Kit which combines concentration, extraction and purification into a single workflow (Cat #R2042, Zymo Research, USA). The viral particles were concentrated from 40 mL of wastewater by precipitation, adding 2.8 mL of Water Concentrating Buffer^{TM} followed by centrifugation at 3 000 × *g* for 15 minutes. After centrifugation, the supernatant was removed, leaving approximately 250 µL of pellet in accordance with the manufacturer's instructions. The pellet was then resuspended using 750 µL of DNA/RNA Shield^{TM}, mechanically lysed (bead beater homogenizer), then centrifuged at 12 000 × *g* for 2 minutes and 400 µL of the supernatant was extracted. After purification of the product using the Zymo-Spin IC Column, elution was performed by adding 30 µL of DNA/RNA-free water to the column matrix and centrifuged. The eluted nucleic acids were stored at -80°C upon analysis.

2.1.4. Concentration and extraction procedures evaluation

Analyses were performed on four samples: Lyon on April 2, 2024, April 16, 2024, and May 14, 2024, and Nîmes on March 20, 2024. In accordance with the procedures described in §2.1.3 sample concentration and nucleic acid extraction, samples were either (i) extracted directly from 250 µL of wastewater without a concentration step, or (ii) concentrated from 5 mL or 40 mL of wastewater prior to the nucleic acid extraction step. Each extraction was performed in triplicate to assess repeatability. Recovery rate was calculated as follows: ([5 mL or 40 mL in gc/L]/[250 µL in gc/L] × 100).

2.2. Assay development

2.2.1. Primers and probes selection

Following the aforementioned World Health Organization recommendations and in order to avoid false positives, a one-step multiplex RT-dPCR assay was developed to simultaneously quantify three distinct measles virus gene regions —N (nucleocapsid), P (phosphoprotein) and M (matrix) genes— using S1, S2 and S3 systems, respectively. The design of the S2 and S3 systems, targeting P and M genes, was evaluated using a total of 22 complete genomes representing the D8, B3, and H1 genotypes of measles, which were aligned using BioEdit software (Version 7.2.6; (Hall, 2011)). After identifying the regions of interest, Primer III software (Version 4.1.0; (Untergasser et al., 2012)) was used to design 12 amplification systems that meet the required criteria. These conditions were established by comparison with the system used by the French NRC for Measles, whose efficiency in dPCR had already been validated. A pair of primers and a Taqman probe targeting the phosphoprotein and matrix were selected from these amplification systems. All hydrolysis probes used in S1, S2 and S3 systems were designed with a 6-carboxyfluroscein (FAM) or hexachlorofluorescein (HEX) or Cyanine 5 (Cy5) fluorophore and ZEN/Iowa Black (IWA) quenchers for more efficient quenching (IDT - Integrated DNA Technologies, USA). In this study, the S2 and S3 systems targeting the P and M genes, respectively, were considered as confirmation targets for positive wastewater samples.

As the vaccine strain can be excreted in the urine of recently vaccinated persons and potentially lead to incorrect conclusions, a specific digital RT-dPCR that can detect and exclude these types of vaccine particles was used. Primers and probes specifically targeting the nucleocapsid of the attenuated vaccine strains (genotype A), called "VS system", were designed and tested by the French NRC for Measles.

An *in-silico* analysis of S1, S2, S3 and VS systems, designed and used in this study, was performed using BLAST queries (Altschul et al., 1990), with analysis performed in October 2024. The data obtained clearly demonstrated that there was neither cross-amplification nor non-specific amplification, confirming the specificity of each system developed. The different sets of primers and probes used are presented in Table 1.

2.2.2. Positive controls for sensitivity, repeatability, reproducibility and multiplexing evaluation

An ultramer RNA oligonucleotide was constructed as positive control to detect and quantify the measles nucleocapsid gene, in accordance with WHO recommendations. It consists of a target sequence derived from the measles virus genotype D8 strain PP556758.1 (5'-

Table 1
Primers and probes used in this study.

System	Primer or Probe	Sequence (5'-3')	Targeted Gene	Reference
S1, Measles	MVN1139Fw	TGGCATCTGAACTCGGTATCAC	Nucleocapsid	(Hummel et al., 2006)
	MVN1213Rv	TGTCCTCAGTAGTATGCATTGCAA	(N)	
	MVN1163probe	CCGAGGATGCAAGGCTTGTTTCAGA (FAM/IWA)		
S2, Measles	MVP1921Fw	ATATCAGACAACCCAGGACA	Phosphoprotein (P)	This work
	MVP2016Rv	GATCCAATTGCTGAGAGGC		
	MVP1973Sd	CAGTTCGGGTCTCAGCAAAC (HEX/IWA)		
S3, Measles	MVM3718Fw	CCACTGAGCTTGACATAGTT	Matrix	This work
	MVM3813Rv	CCAAGGTGTGAGGAGAGTTA	(M)	
	MVM3744Sd	CGTACAGCAGGGCTCAATGA (Cy5/IWA)		
VS, Vaccine strain	MeVN1292Fw(A)	CAGAGATTGCAATGCATACTACTGA	N	This work
	MeVN1366Rv(A)	CTCACTTTGATCACCGTGTAG	(specific to the vaccine strain)	
	MeVN1320Probe(A)	CAAGATCAGTAGAGCGGTTGG		
FRNAPH-II	VTB4-F-GIIpro	CACTCGCGATTGTGCTGTCCGATT		(Wolf et al., 2010)
	VTB4-F-GIIf	ACCTATGTTCCGATTCASAGAG		
	VTB4-F-GIIr	GGTAGGCAAGTCCATCAAAGT (FAM/BHQ1)		
Feline Calicivirus (FCV-F9)	FCVr	GATCGGAAAAGTAACGAAGGATGT		(Gassilloud et al., 2003)
	FCVf	GATAGCCCCAGCGRCGAAG		
	FCVp	TCGACCCAATCGCCTCGTGTCA (Cy5.5/IWA)		

TGGCATCTGAACTCGGTATCACTGCCGAGGATGCAAGGCTTGTTTCA-GAGATTGCAATGCATACTACTGAGGACA-3'), preceded by a tag sequence (5'-ACAGGTACGTTAATAGTTAATAGCG-3'). This ultramer was produced and quantified at 8.40×10^{12} gc/µL by IDT, and at 6.90×10^{13} gc/µL using the Qubit RNA BR Assay (Qubit™ Fluorometer, Thermofisher) following the manufacturers recommendations. A serial dilution of the ultramer RNA oligo was used to test the efficiency, sensitivity and repeatability of the single RT-dPCR.

The development of multiplex RT-dPCR and its evaluation were performed using six clinical samples of measles virus genotype B3 (n=3) and D8 (n=3), selected based on their incidence in the infected population. These viruses, isolated from human biological samples, are presented in the Table 2. Nucleic acid extraction was performed twice using the QIAamp Viral RNA Mini Kit (Qiagen, France) according to the manufacturer's instructions. The initial sample volume was 10 µL of the first extraction completed to 100 µL with DNAse RNAse free water, and the final elution volume was 50 µL. Extractions were stored at -80°C until analysis.

2.3. RT-dPCR assays and controls

2.3.1. Genome quantification

Amplification was performed using the One-Step RT-ddPCR Advanced Kit for Probes (Bio-Rad, Laboratories, Inc. USA). Reactions were prepared according to the manufacturer's instructions. The reaction, with a final volume of 22 µL, contained 5.5 µL of Supermix 4X, 1.1 µL of DTT (dithiothreitol) 300 mM, 2.2 µL of the Reverse Transcriptase (200 UI), and 1.0 µL of each system, for final primer and probe concentrations of 900 nM and 300 nM, respectively (Table 1), as well as 5.0 µL of extracted RNA. Positive control and no template control (NTC) with DNAse and RNAse free water were included in each series. 20 µL of the total reactional volume were used for the automated droplet generation. Amplification was performed using a T100 thermal cycler (Bio-Rad Laboratories, Inc. USA) with the following conditions: reverse transcription at 50°C for 60 min, polymerase activation at 95°C for 10 min, followed by 40 cycles of denaturation at 94°C for 30 s and annealing/extension at 55°C for 1 min. Final steps consisted of a polymerase deactivation at 98°C for 10 min, followed by a droplet stabilization step at 4°C for 30 min. A temperature ramp of 2°C/s was set for all PCR steps. The samples were analyzed using the QX600 Droplet Reader (Bio-Rad Laboratories, Inc. USA), where the fluorescence signal was measured in the FAM/HEX/Cy5 channels depending on the target. The absolute copy number of each target was calculated using the QX Manager Software 2.0 Standard Edition (Bio-Rad Laboratories, Inc. USA), where thresholding was performed manually at the lowest amplitude that captures true positive clusters based on the signals from negative control and positive control samples. Samples were analyzed when at least 10 000 droplets were generated. Concentration was expressed in cg/µL of analyzed matrix template or cg/L of wastewater (cg/µL of analyzed template × total elution volume in µL / mL of concentrated-extracted wastewater × 1 000).

Table 2
Description of sample genotypes.

Sample ID	Measles Genotype	Strain Name	WHO Strain Designation
1	B3	MVs/Manchester.GBR/44.23	MVs/Lyon.FRA/10.24/6
2	D8	MVs/Oita.JPN/30.22	MVs/SaintPeray.FRA/38.23
3	D8	MVs/Oita.JPN/30.22	MVs/Soyons.FRA/40.23
4	D8	MVs/Victoria.AUS/39.22	MVs/Lyon.FRA/07.24/3
5	B3	NA (not applicable)	MVs/Marseille.FRA/08.24
6	B3	NA	MVs/SaintPierreReunion.FRA/5.19

2.3.2. Process and inhibition controls

F-specific RNA bacteriophages from subgroup II (FRNAPH-II), widely used as specific human fecal indicators in wastewater samples, were used as internal process controls for both the concentration and extraction steps. Samples were diluted 100-fold prior their quantification using the primers and probes listed in Table 1 in accordance with the recommendations described in §2.3.1 genome quantification. As the amounts of FRNAPH-II normalized to N-NH_4 are relatively stable, samples with ratios 10 times lower than the usual value for each city were considered to have experienced a problem during the concentration/extraction steps.

In addition, the feline calicivirus F9 (FCV-F9) genome was used to assess possible reaction inhibition due to wastewater compounds and/or extraction residues co-extracted in the samples. A known quantity of FCV-F9 genome was added directly to the master mix, and a percentage of inhibition was calculated by comparing the FCV-F9 genome copies obtained in the samples with those in the blank reactions performed with DNAse RNAse free water (percentage of inhibition = 1 - ([sample]/[blank]) × 100; concentrations given in copies/L).

2.4. Epidemiological data

Measles has been a notifiable disease in France since 2005. Clinicians and microbiologists must report suspected or confirmed cases to regional public health authorities. Case confirmation is based on biological or epidemiological criteria (e.g., a clinical case linked to a laboratory-confirmed case). Data are then collected and analyzed by the National Public Health Agency. The Lyon and Nîmes sites were selected based on evidence of viral circulation in 2024. Maxéville was included as background control, given that only a single case was reported during the same period, without any identified clusters.

2.5. Statistical analysis

Statistical analysis was performed using Jamovi Version 2.3.28.0 ("The jamovi project," 2024).

3. Results

3.1. RT-dPCR efficiency, sensitivity and repeatability of the reference system

The efficiency of amplification was assessed using an ultramer RNA oligo produced by IDT, with the WHO primers and probe set targeting the N gene and forming the reference system designated "S1". A concentration of 1.0×10^{13} gc/µL was obtained by RT-dPCR with a coefficient of variation (CV) of 28% (n=11). As this value falls between 8.4×10^{12} gc/µL as determined by IDT and 6.9×10^{13} gc/µL as determined using a Qubit procedure, amplification using the S1 system can be considered efficient. Following this, the sensitivity of the S1 system was evaluated using both the ultramer RNA oligo and the measles genome B3 (Sample 1, Table 2). The limit of quantification was determined to be 1.5 gc/µL with a CV of 28% (n=11) and 1.7 gc/µL with a CV of 26% (n=10), respectively.

3.2. Multiplex RT-dPCR: S1, S2 and S3 comparison

Single, dual and triplex RT-dPCR were performed to compare the results obtained on the same genomes (viral extracts from genotypes B3 and D8). The comparison of copies/µL obtained from systems 1 to 3, multiplexed or not, is summarized in Table 3. These results highlight that there was no difference between the single-plex and the triplex in terms of quantification, regardless of the system considered (Kruskal-Wallis test, P=0.925).

In order to evaluate the reproducibility of the triplex, samples 1 to 6 were measured three times (Table 4). The results showed that the measurements were not affected by the target (Kruskal-Wallis test, P=0.188), and a CV was calculated for the different strains used. The lowest CV was obtained for sample 1 with the highest concentration (mean of 16.4 copies/µL) while the highest CV was obtained for sample 6 with the lowest concentration (mean of 1.9 copies/µL). For others samples, the CV ranged from 21% to 31%.

Table 4
Evaluation of the triplex's reproducibility (n=3). The mean, standard deviation (SD) and coefficient of variation (CV) were calculated from the values obtained in copies/µL for samples 1 to 6.

	System 1 (N)	System 2 (P)	System 3 (M)	Mean	SD	CV %
Sample ID1	15.7	17.4	16.7	16.4	1.7	10%
	18.2	18.8	13.6			
	15.7	16.9	14.8			
Sample ID2	9.4	8.8	4.9	8.6	2.6	30%
	13.6	9.9	5.4			
	9.2	8.5	7.9			
Sample ID3	6.4	2.3	2.7	4.0	1.3	31%
	3.5	4.4	3.2			
	5.1	4.1	4.7			
Sample ID4	8.6	6.4	5.1	7.4	1.6	21%
	9.9	7.6	7.3			
	9.0	5.7	6.8			
Sample ID5	8.4	5.1	2.9	5.5	1.7	31%
	8.1	4.2	5.3			
	5.3	5.5	4.8			
Sample ID6	3.6	2.2	2.2	1.9	0.9	47%
	1.5	1.1	0.4			
	2.4	1.9	2.3			

3.3. Sensitivity comparison between the triplex and attenuated vaccine strain systems

As the attenuated vaccine strains can be excreted in urine, they may be found in wastewater. In order to exclude "measles vaccine positive samples", primers and probes specifically targeting the nucleocapsid of the attenuated vaccine strain (referred to as the "VS system" in this study) were designed and tested by the NRC.

To compare sensitivity, duplicate quantifications were performed using different dilutions of the attenuated vaccine strains as a matrix template with both systems (VS and triplex), and selectivity was assessed using samples 1 to 6 (Table 5). According to the data obtained, the VS system targets only the attenuated vaccine strains, as expected, and the sensitivity of VS and triplex systems is comparable, except of S3. As the concentrations obtained with S3 were 2 to 3 times lower than those of the other targets, this could be used to infer the presence of the attenuated vaccine strains in a sample.

3.4. Limit of blank (LoB)

The limit of blank (LoB) is one of the most important parameters to

Table 3
Comparison of copies/µL obtained from systems 1 to 3, whether included in the singleplex, the duplex or the triplex, and using samples 1 to 6 as templates.

Target / Multiplexed status	Copies/µL					
	Sample ID1	Sample ID2	Sample ID3	Sample ID4	Sample ID5	Sample ID6
System 1 (N)						
Singleplex S1	15.7	9.2	5.1	9.0	5.3	2.4
Duplex S1-S2	20.6	9.4	4.9	7.8	7.0	2.0
Duplex S1-S3	15.3	11.0	5.1	9.5	6.4	2.3
Triplex	15.7	9.4	3.5	9.9	8.1	3.6
System 2 (P)						
Singleplex S2	16.9	8.5	4.1	5.7	5.5	1.9
Duplex S1-S2	15.4	8.1	3.5	5.1	5.3	3.5
Triplex	17.4	8.8	4.4	7.6	4.2	2.2
System 3 (M)						
Singleplex S3	14.8	7.9	4.7	6.8	4.8	2.3
Duplex S1-S3	14.5	10.0	4.5	7.7	4.0	1.5
Triplex	16.7	4.9	3.2	7.3	5.3	2.2

Table 5
Sensitivity comparison between the triplex (systems 1 to 3) and the attenuated vaccine system (VS), with selectivity confirmation using the attenuated vaccine genome (Vg) and Samples ID 1 to 6 as models. Quantifications were performed in duplicate (n=2), except where marked "*" (n=1). Results are expressed in copies/µL.

		Singleplex	Triplex		
		VS (N)	System 1 (N)	System 2 (P)	System 3 (M)
Vg	1	134.2*	173.8*	158.8*	53.7*
	1/10	14.1	16.0	11.3	5.0
	1/20	5.3	8.3*	9.3*	2.3*
	1/40	3.2*	6.5	3.5	0.9
	1/80	1.8	1.3*	1.0*	0.3*
	1/160	0.4	0.4	0.4	0.2
Samples IDs 1 to 6		Not detected	Detected		

define first to exclude "false-positive" results and secondly to determine the detection and quantification limits. As absolute quantification by dPCR follows the Poisson distribution, samples with the same number of positive droplets but different total droplet counts, will yield slightly different concentrations. Therefore, in this work, the LoB, limit of detection (LoD), and limit of quantitation (LoQ) were determined based on the number of positive droplets. The LoB was defined as the highest number of positive droplets observed in target-free samples. The LoD was subsequently determined as the LoB plus one positive droplet, and the LoQ was defined as the LoD plus one positive droplet.

For each triplex target, the number of positive droplets corresponding to the LoB was determined using DNAse RNAse free water (n=10) and wastewater samples from Maxéville WWTP (n=14), presumed free of measles genome. For the attenuated vaccine strains, the LoB was determined using biological samples, DNAse RNAse free water (N=13) and wastewater samples from Lyon and Nîmes WWTPs (n=20). The data obtained, listed in Table 6, allowed the LoB to be set at 0 for the vaccine strain, and 1 for systems 1 to 3. Consequently, the LoD was defined as 1 positive droplet for the attenuated vaccine, and 2 positive droplets for systems 1 to 3, and the LoQ as 2 and 3 positive droplets respectively.

3.5. Concentration and extraction recovery procedure

Concentration and extraction recovery were assessed by quantifying group II RNA-specific bacteriophages (FRNAPH-II), a target known to be naturally abundant in raw urban wastewater and present at high concentrations, unlike measles. Four samples from WWTPs in Nîmes (March 20, 2024) and Lyon (February 2, 2024; April 16, 2024; May 12, 2025) were extracted in triplicate, either directly from 250 µL, or from 5 mL or 40 mL after a concentration step. The results of these analyses, presented in Fig. 1, showed that the highest concentrations were obtained from 250 µL wastewater samples without a concentration step (Kruskal-Wallis test, $P<0.001$). No significant difference was observed between the concentrations obtained for 5 mL or 40 mL samples (Dwass, Steel, Critchlow et Fligner test, $P=0.163$). Their coefficients of variation, listed in Table 7, ranged from 8% to 14% for the 250 µL condition, 5% to 12% for the 5 mL condition and 2% to 16% for the 40 mL condition. Recovery was calculated assuming 100% efficiency for the "250 µL condition", and the results for each WWTP/condition are presented in Fig. 2. Recovery rates were significantly higher from 5 mL samples (Student test, $P=0.011$).

Table 6
LoB, LoD and LoQ —marked in bold— for each system of the triplex (Systems 1 to 3) and the attenuated vaccine strain control system, in number of positive droplets. The number of replicates per system/template is indicated in the "N" column. The no-template control (NTC) was performed using DNAse/RNase-free water.

			Positive droplets				
Systems	**Template**	**N**	**Min.**	**Max.**	**LoB**	**LoD**	**LoQ**
System 1	NTC	10	0	0	0	1	2
	Maxéville WWTP	14	0	1	**1**	**2**	**3**
System 2	NTC	10	0	1	**1**	**2**	**3**
	Maxéville WWTP	14	0	1	**1**	**2**	**3**
System 3	NTC	10	0	0	0	1	2
	Maxéville WWTP	14	0	1	**1**	**2**	**3**
Vaccine strain	Samples 1 to 6, and NTC	13	0	0	**0**	**1**	**2**
	Environmental samples	20	0	0	**0**	**1**	**2**

The LoB, LoD and LoQ values used were determined on the basis of the highest number of positive droplets in matrices free of measles contamination.

3.6. Quantification of measles genome in raw urban wastewater samples

Quantification of measles genome in wastewater samples collected from Lyon (n= 18) and Nîmes (n= 22), was performed using the validated triplex RT-dPCR assay targeting the nucleocapsid, phosphoprotein and matrix *via* systems S1, S2 and S3, respectively. In general, samples were considered positives if at least two triplex targets had a minimum of 2 positive droplets (i.e., LoD). However, as shown in Supplementary Table 2 and detailed in Fig. 3, some samples had: (i) only one target of the triplex reaching at least the LoQ; (ii) S2 and S3 reaching at least the LoD, but not the reference system; or (iii) one positive droplet in each system of the triplex. Based on the results observed across the three targets, two confidence levels were established in Table 8.

On the one hand, a confidence level of 1 corresponds to a suspected positive result. This includes samples with one target at or above the LoD, or with a positive droplet detected in each target of the triplex. Based on the data in Table 6, such an event could occur at a rate of 0.072%, which remains below the usual 5% error threshold. Nevertheless, these samples may need to be reanalyzed by increasing the sample input volume or re-extracted using a larger volume of wastewater. On the other hand, a confidence level of 2 corresponds to true positives, which includes: (i) samples with systems S2 and S3 at the LoD, but not the reference system; (ii) samples with systems S1 and either S2 or S3 at least at the LoD; or (iii) samples with all systems at the LoD. Finally, a sample is considered "not detected" under our analytical conditions, if it does not meet any of the conditions described in Table 8.

In line with these confidence levels, 18 samples (45%) were considered positives (Fig. 3), including 12 from Lyon and 6 from Nîmes (Supplementary Table 2). Among these, 11% (2/18) had a confidence level of 1, and 89% (16/18) a level of 2. All positive samples tested negative for the VS system. None of the tested samples —including the "not detected" ones— were considered positive for inhibition (FCV-F9 recovery rate, 1.07 ± 0.24), nor did they encounter any problems during the concentration/extraction steps (mean FRNAPH-II concentration: 3.01×10^7 gc/L $\pm$ 1.75 gc/L of wastewater analyzed). Among positive samples from Lyon, concentrations ranged from 5.8×10^2 copies/L and 4.6×10^3 copies/L, with an average concentration of 2.7×10^3 copies/L, 2.1×10^2 copies/L and 2.6×10^3 copies/L for systems S1, S2 and S3, respectively. For Nîmes, concentrations ranged from 1.8×10^3 copies/L to 2.2×10^4 copies/L, with an average concentration of 6.5×10^3 copies/L, 5.0×10^3 copies/L and 5.6×10^3 copies/L for systems S1, S2 and S3, respectively.

3.7. Comparison with measles cases

For each positive sample, the average viral load was calculated and normalized relative to $N\text{-}NH_4$ to account for fluctuations in population size and/or meteorological conditions. This data, combined with reported measles cases – categorized into two age groups (under 3 years and over 3 years) – is presented in Fig. 4. In the Lyon metropolitan area, 98 confirmed clinical cases were reported between weeks 3 and 24 of 2024 (based on the week of rash onset), including 42 cases in children under 3 years of age. In this age group, urine is more frequently disposed

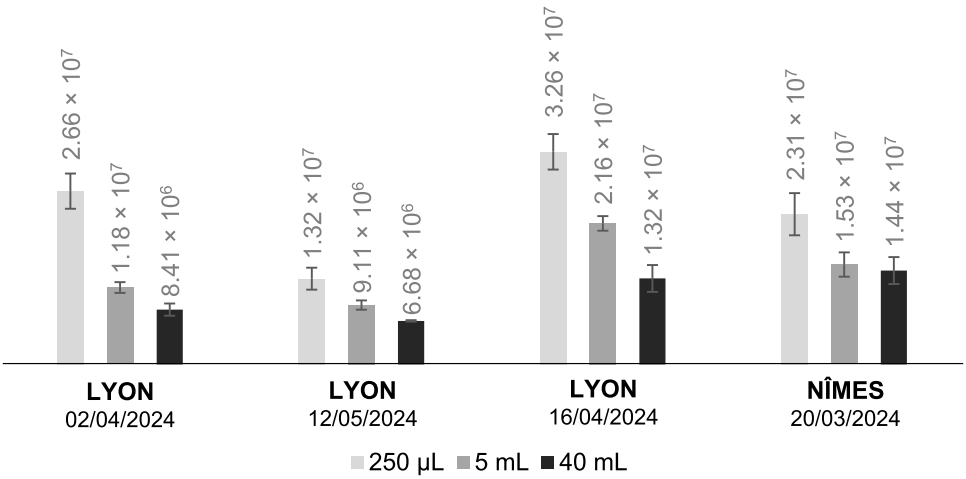

Fig. 1. Concentration of F-specific RNA bacteriophages from genogroup II in copies/L of raw urban wastewater from Lyon and Nîmes WWTPs (n=3), according to the volume of extracted samples.

Table 7
Coefficient of variation determined from samples extracted in triplicate from either 250 µL, 5 mL, or 40 mL of wastewater.

	250 µL	5 mL	40 mL
Lyon - 02/04/2024	10%	7%	11%
Lyon - 12/05/2024	13%	8%	2%
Lyon - 16/04/2024	8%	5%	16%
Nîmes - 20/03/2024	14%	12%	14%

of with household waste, potentially limiting its detection in wastewater. Nevertheless, this remains an important factor to consider, as close family members may be infected without being diagnosed or officially reported. The highest number of clinical cases was recorded in week 13 (n= 12), with half occurring in children over 3 years of age. When considering only cases in this age group, the peak (n=7) occurred twice: first in week 12 and then again in week 18.

Surveillance of measles virus in wastewater began in week 11, as no samples were available prior to this date. The first positive sample was detected in week 12. A first peak was observed in week 13 (134 copies/L, normalized to N-NH4), followed by two another in weeks 18-20 (approximately 195 copies/L, normalized to N-NH4), after which the concentration decreased. A positive correlation was visually observed between the rise in reported measles cases and the increase in measles virus concentrations in wastewater. However, this correlation could not be demonstrated at the Nîmes station, where 17 of the 18 reported cases (from week 3 to week 28) involved children under 3 years of age. In addition, the highest concentration (419 copies/L, normalized to N-NH4) was recorded in week 7. Contrary to expectations, no clinical cases were reported during that period. Since the sample was taken during school holidays, the increase observed in week 7, followed by a decrease in the measles genome concentration, may be explained by population movement during that period.

4. Discussion

In this study, we developed and validated a one-step multiplex RT-dPCR assay targeting three distinct regions of the measles virus genome currently circulating the French population, in order to detect the virus in samples collected at the inlet of WWTPs. This triplex test included the WHO reference system targeting the nucleocapsid gene, used for clinical screening of measles by the French NRC for Measles, as well as the phosphoprotein and matrix genes. A multi-target approach was essential for several reasons: (i) it aligns with current SARS-CoV-2 WWS strategies (Jourdain et al., 2025) as well as European Commission recommendations (The European Commission, 2021); (ii) it reduces false positives by confirming initial detections with additional targets, thereby enhancing robustness; and (iii) it mitigates the risk of false negatives caused by viral genome mutations, as demonstrated for measles (Beck et al., 2024) and SARS CoV-2 (Wang et al., 2020; Ziegler et al., 2020). Such a strategy also seemed relevant, as virus genotyping from wastewater samples remains a major challenge, mainly due to the low target concentrations and the complexity of the environmental matrix (Price et al., 2024; Wade et al., 2022). To further avoid false

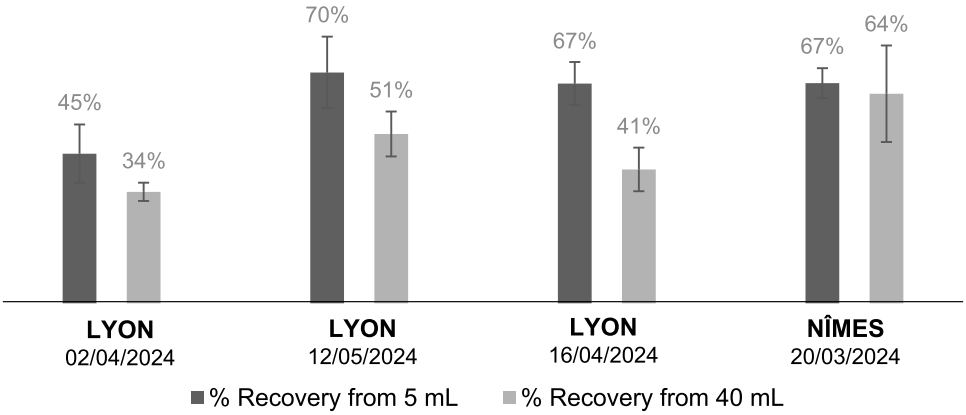

Fig. 2. Recovery determined for FRNAPH-II from samples extracted in triplicate from 5 mL or 40 mL of raw urban wastewater (n=3).

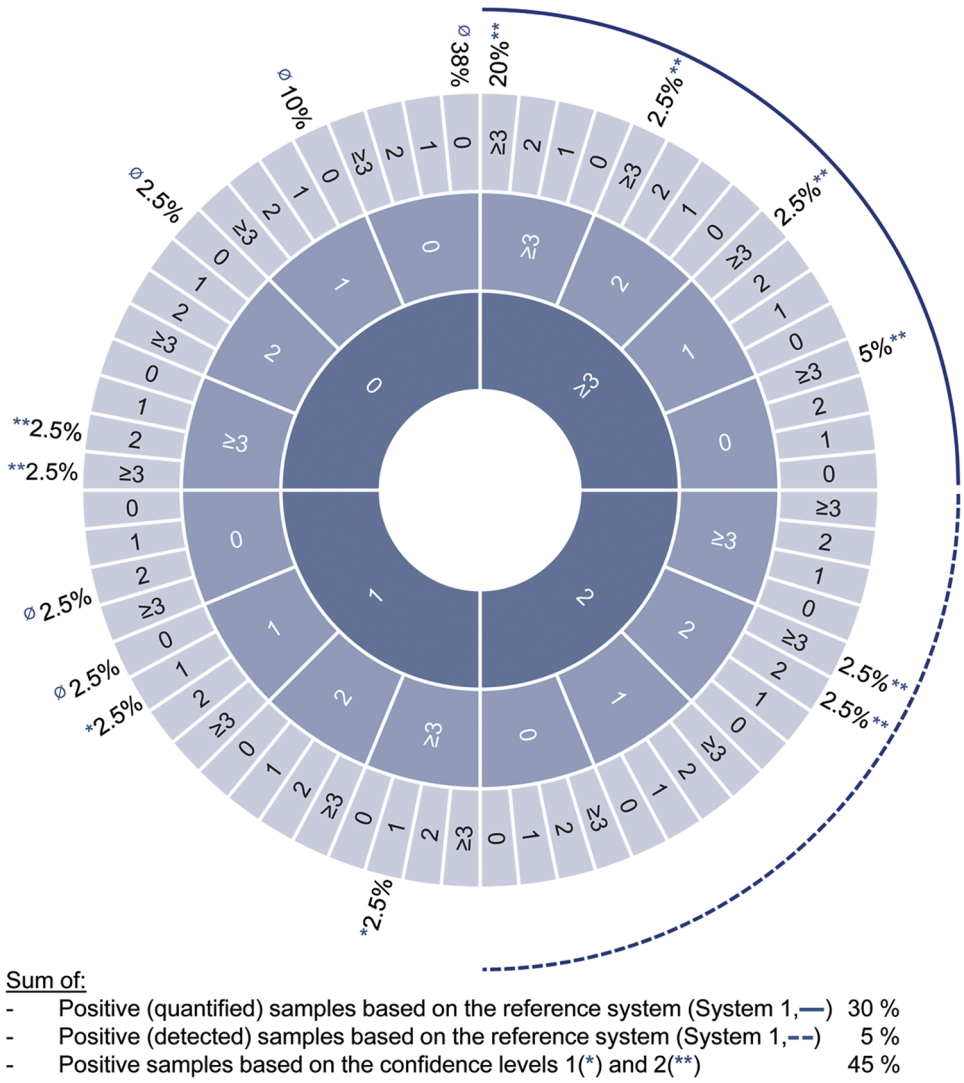

Fig. 3. Percentage of samples meeting each condition, based on the number of droplets in each triplex system —in dark blue-grey for System 1, medium blue-grey for System 2 and light blue-grey for System 3. Samples were categorized as undetected (Ø), suspected positives with a confidence level of 1 (*), or confirmed positives with a confidence level of 2 (**).

Table 8
Description of confidence level assigned status and examples for each condition.

Confidence level	Condition	Status	*Example Repartition for positive droplets*		
			S1	***S2***	***S3***
1	S1, S2 or S3 at or above the LoQ	Suspected positive	*0*	*≥3*	*0*
	OR at least one positive droplet in each target of the triplex		*1*	*1*	*1*
2	S2 and S3 systems at or above the LoD	Positive	*<2*	*≥2*	*≥2*
	S1 and S2 system at or above the LoD		*≥2*	*≥2*	*<2*
	S1 and S3 system at or above the LoD		*≥2*	*<2*	*≥2*
	S1, S2 and S3 systems at or above the LoD		*≥2*	*≥2*	*≥2*

positives, we analyzed the limit of blank, a crucial step in determining interpretation threshold (Table 8). Positive samples were also confirmed using a vaccine strain detection system, as recently vaccinated individuals may excrete the vaccine strain in their urine. It is important to distinguish between vaccine viruses and wild-type viruses, as the presence of vaccine viruses in wastewater due to human shedding may hinder data interpretation. By ensuring an accurate and easy differentiation between measles strains, public health measures can be appropriately targeted, avoiding misinterpretation and enabling precise actions based on true measles activity within a population and geographical area.

To minimize the risk of false-negatives, FRNAPH-II was used as a process control, while FCV-F9 served as inhibition control, demonstrating minimal matrix effects on detection (average recovery rate of 1.07 ± 0.24). RT-dPCR also proved advantageous for measles quantification, as it is less susceptible to inhibitory molecules commonly found in wastewater, which can interfere with enzymatic reactions during reverse transcription and amplification (Ahmed et al., 2022; Ciesielski et al., 2021; Rački et al., 2014).This method allows quantification without the need for a calibration curve, according to a mathematical law based on the number of partitions (Group and Huggett, 2020). Moreover, nucleic acids purification at the last step of extraction further reduced inhibition, enabled a reduction in elution volume (thereby increasing the final concentration), and improved the probability of detection. This system is currently used to monitor other pathogens in

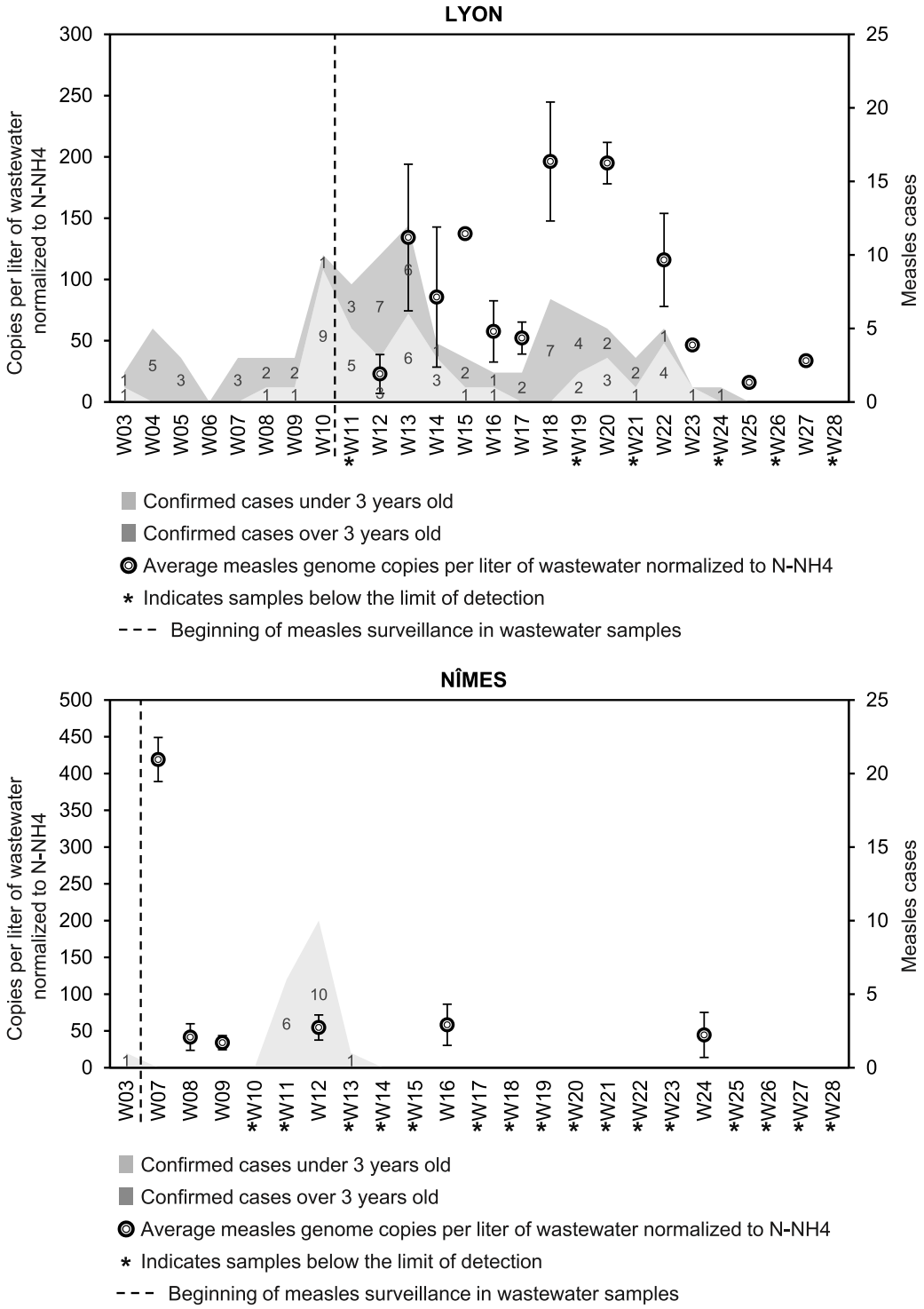

Fig. 4. Evolution of measles concentration (average of the triplex in gc/L of analyzed wastewater, normalized to N-NH_4) over time in Lyon and Nîmes. Also shown are the number of notified measles cases (by week of rash onset) in children under 3 years old (light grey), and in individuals over 3 years old (dark grey). The dotted line delimits the start of samples available for measles extraction and quantification. Below the LoD limit, samples were marked as not detected (*).

wastewater, including SARS-CoV-2.

Wastewater samples with volumes from 5 mL to 40 mL were tested for measles virus. The recovery rate of the method, measured using naturally occurring F-specific RNA bacteriophages, varied between 0.45-0.70 and 0.34-0.65, depending on the volume. Using 40 mL improved the probability of detection. Our molecular approach confirmed the feasibility of monitoring measles virus in wastewater from areas where genotypes B3 and D8 are circulating. Measured concentrations were low for all targets, ranging between 5.7×10^2 gc/L – 4.6×10^3 gc/L and 1.8×10^3 gc/L – 2.2×10^4 gc/L, depending on the area considered. Despite the low values observed, viral loads detected in both areas (Supplementary Table S2) suggest that previous estimates of total daily measles RNA excretion per infection are underestimated (Chen and Bibby, 2023). Our findings suggest that measles virus shedding likely falls within the upper range of the proposed distribution, highlighting the need for more accurate quantification of excretion

dynamics and reinforcing the value of WWS, even in low-incidence settings. Ammonium nitrogen was used as a normalization factor due to its biological relevance for measles (a urine-excreted virus), its widespread availability in WWTP routine monitoring, and its use in the national SARS-CoV-2 wastewater surveillance system in France (Jourdain et al., 2025). While non-domestic inputs may influence ammonium levels and vary between sites, temporal analyses were conducted independently at each site. We therefore assume that such contributions remain relatively stable over time, limiting their impact on trend interpretation.

While our main objective was to develop and validate the method under field circulation conditions, our results underscore the potential of WBE as a complementary tool to clinical surveillance. As dPCR demonstrates greater resilience to inhibitors compared to real-time PCR (Rački et al., 2014), which is particularly advantageous when analyzing complex matrices such as wastewater, and its absolute quantification eliminated the need for standards —thereby simplifying the workflow and reducing variability— this approach can be considered cost-effective. This is especially true when dPCR infrastructure is already available in the laboratory, further facilitated by its efficient implementation of multiplex assays. It also provides a non-invasive and unbiased method for tracking viral circulation at the population-level, thereby supporting public health decision-making. This is particularly relevant in the current context of disease resurgence (Mahase, 2025). Measles WWS could serve as a complementary detection system to identify outbreaks, as demonstrated in South Africa, where WWS detected the measles virus in 48% of cases where clinical surveillance had failed (Ndlovu et al., 2024). However, implementing systematic nationwide WWS would require substantial sampling efforts and may have limited capacity to detect small, localized clusters due to the disease's low incidence in France — unless the cluster occurs within the population served by the WWTP, as was the case in Nîmes. Nevertheless, WWS could be valuable for tracking the spatial and temporal spread of the virus within the population, especially when circulation levels are high enough that epidemiological links between clusters become challenging to establish. Targeted WWS could be beneficial in areas with low vaccination coverage (Benschop et al., 2017), during high-risk events (Toro et al., 2024), or in underserved populations with limited access to healthcare and underdiagnosis. It could also support disease elimination efforts by detecting low-level residual circulation that might go undetected during clinical surveillance in countries close to elimination status. Indeed, the situation observed in Nîmes may illustrate this possibility. While it remains speculative to directly link the January 2024 case with the June 2024 pediatric cluster in the absence of genomic sequences from wastewater samples, we cannot rule out the presence of undetected low-level circulation between these events, potentially identifiable through WWS.

Several factors can influence the interpretation of WWS data, affecting its reliability and accuracy. It is essential to understand these limitations in order to implement effective public health measures and allocate resources appropriately. Genomic concentrations in wastewater are influenced by geographical, climatic, socio-economic, and infrastructural factors (Price et al., 2024; Wade et al., 2022) as well as the epidemiological context (Chen and Bibby, 2023). Consequently, direct comparisons between sites are challenging. In this study, genomic concentration levels varied, with a higher signal observed in Lyon than in Nîmes. This may be explained by the higher number of cases and the age distribution of those infected, even though the population connected to the Lyon WWTP serves a significantly larger population than Nîmes, which could lead to signal dilution. In Nîmes, all cases involved infants likely still using diapers, which limits the excretion of viral particles -primarily shed in urine- into the wastewater system, although not excluding it completely (e.g., during bathing). Given the low incidence of the disease, a robust analytical strategy combining multiple targets is necessary, with careful evaluation considering the epidemiological context. Confidence levels were proposed to support the interpretation of results. Relevant analytical results should also be considered. For instance, the positive predictive value increases with incidence. A confidence level 1 result (suspected positive) is more likely to be a true positive during transmission episodes or after previously confirmed detections (confidence level 2). Furthermore, RNA can degrade throughout the whole process, which may lead to underestimates, particularly in retrospective sample analysis. Finally, it is essential to consider the possibility of detecting vaccine strains and to account for the potential evolution of the virus. This requires a continual re-evaluation of detection systems, and the integration of a vaccine-strain specific detection system, to mitigate confounding factors, such as vaccine shedding in wastewater.

5. Conclusion

The analytical strategy developed in this study, which combines triplex dPCR and vaccine strain control systems, was validated here for the first time for application to wastewater surveillance. This multiplex approach demonstrated the feasibility of reliably detecting and quantifying measles virus in wastewater, including in low-concentration conditions and during retrospective analyses performed in the context of real viral circulation. The use of a multiplex strategy for this surveillance offered multiple advantages. Notably, it improves the ability to confirm of measles virus circulation, particularly when concentrations are too low to characterize circulating strains by genomic sequencing. It also reduced the risk of false negatives due to mutations that might interfere with the enzymatic reverse transcription and amplification steps. Further, the control system enabled the distinction between vaccine strains and wild-type viruses, thus enhancing data interpretation.

The deployment of RT-dPCR was based on the analysis of samples collected at the inlet of wastewater treatment plants through 24-hour composite sampling, providing a cost-effective, non-invasive method for tracking measles virus circulation within communities over time, while significantly limiting the number of analyses required. As such, wastewater-based epidemiology (WBE) represents a valuable tool to support virus detection, especially in low-incidence settings, and to complement existing clinical surveillance systems as part of broader measles elimination strategies.

Nonetheless, it remains essential in future studies to acquire complementary data to explore, for example, whether the proposed strategy could provide early warning potential by studying if there is a lag between wastewater detection and reported clinical cases. The multiplex RT-dPCR method presented in this study will be useful for performing such evaluations. It is also important to (i) recognize the method's limitations, (ii) interpret the results with caution, and (iii) emphasize the role of wastewater surveillance.

Funding

The development and assays organized to test the measles strategy are funded by the national funding of the Laboratory of National Reference of the French Agency for Food, Environmental and Occupational Health & Safety and NRC for MMR viruses. Funding is aimed at supporting evaluation of wastewater-based methodologies for tracking pathogens of interest in wastewater.

CRediT authorship contribution statement

Véronica Roman: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Formal analysis, Data curation. **Frédéric Jourdain:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **Emilie Pele:** Investigation. **Elise Brottet:** Writing – review & editing, Investigation. **Anne Guinard:** Writing – review & editing, Investigation. **Damien Mouly:** Writing – review & editing, Investigation. **Sandra Medragh:** Investigation. **Christophe Cordevant:** Writing – review &

editing, Funding acquisition. **Benoît Gassilloud:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization. **Julia Dina:** Writing – review & editing, Writing – original draft, Validation, Supervision, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

The authors would like to thank all participating WWTPs, and partner laboratories from SUM'EAU network for their contributions for sending wastewater samples. We are also grateful to Alison Manele and Anaïs Erbs for their dedicated efforts in conducting wastewater analyses, Laura Zenatti, Sophie Lardy-Fontan and Thierry Chesnot for reviewing; and Laïla Toro for spell- and grammar-check.

Note: The graphical abstract was designed using resources from Freepik.

Data availability statement

The processed data used to generate the results presented above are available in the figures. The raw data—containing detailed information on case age and exact symptom onset dates—cannot be shared due to legal and ethical restrictions. The raw data required to reproduced the above findings linked to the RT-dPCR will be available on request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2025.124379.

References

Ahmed, W., Smith, W.J.M., Metcalfe, S., Jackson, G., Choi, P.M., Morrison, M., Field, D., Gyawali, P., Bivins, A., Bibby, K., Simpson, S.L., 2022. Comparison of RT-qPCR and RT-dPCR Platforms for the Trace Detection of SARS-CoV-2 RNA in Wastewater. ACS ES&T Water 2, 1871–1880. https://doi.org/10.1021/acsestwater.1c00387.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J. Mol. Biol. 215, 403–410. https://doi.org/10.1016/S0022-2836(05)80360-2.

Beck, A.S., Lopareva, E.N., Hwang, H., Hart, D., de Almeida, M., Anderson, R., Rota, P.A., Bankamp, B., 2024. Evaluation of the sensitivity of a measles diagnostic real-time RT-PCR assay incorporating recently observed priming mismatch variants, 2024. Eurosurveillance 29. https://doi.org/10.2807/1560-7917.ES.2024.29.28.2400410.

Benschop, K.S.M., van der Avoort, H.G., Jusic, E., Vennema, H., van Binnendijk, R., Duizer, E., 2017. Polio and Measles Down the Drain: Environmental Enterovirus Surveillance in the Netherlands, 2005 to 2015. Appl. Environ. Microbiol. 83. https://doi.org/10.1128/AEM.00558-17 e00558-17.

Bubba, L., Benschop, K.S.M., Blomqvist, S., Duizer, E., Martin, J., Shaw, A.G., Bailly, J.-L., Rasmussen, L.D., Baicus, A., Fischer, T.K., Harvala, H., 2023. Wastewater Surveillance in Europe for Non-Polio Enteroviruses and Beyond. Microorganisms 11, 2496. https://doi.org/10.3390/microorganisms11102496.

Chen, W., Bibby, K., 2023. Making waves: Establishing a modeling framework to evaluate novel targets for wastewater-based surveillance. Water Res. 245, 120573. https://doi.org/10.1016/j.watres.2023.120573.

Ciesielski, M., Blackwood, D., Clerkin, T., Gonzalez, R., Thompson, H., Larson, A., Noble, R., 2021. Assessing sensitivity and reproducibility of RT-ddPCR and RT-qPCR for the quantification of SARS-CoV-2 in wastewater. J. Virol. Methods 297, 114230. https://doi.org/10.1016/j.jviromet.2021.114230.

Eckerle, I., Keller-Stanislawski, B., Santibanez, S., Buderus, S., Hillmann, M., Drosten, C., Eis-Hübinger, A.M., 2013. Nonfebrile Seizures after Mumps, Measles, Rubella, and Varicella-Zoster Virus Combination Vaccination with Detection of Measles Virus RNA in Serum, Throat, and Urine. Clin. Vaccine Immunol. 20, 1094–1096. https://doi.org/10.1128/CVI.00084-13.

Farkas, K., Pellett, C., Alex-Sanders, N., Bridgman, M.T.P., Corbishley, A., Grimsley, J.M. S., Kasprzyk-Hordern, B., Kevill, J.L., Pântea, I., Richardson-O'Neill, I.S., Lambert-Slosarska, K., Woodhall, N., Jones, D.L., 2022. Comparative Assessment of Filtration- and Precipitation-Based Methods for the Concentration of SARS-CoV-2 and Other Viruses from Wastewater. Microbiol. Spectrum 10, e01102–e01122. https://doi.org/10.1128/spectrum.01102-22.

Gassilloud, B., Schwartzbrod, L., Gantzer, C., 2003. Presence of Viral Genomes in Mineral Water: a Sufficient Condition To Assume Infectious Risk? Appl. Environ. Microbiol. 69, 3965–3969. https://doi.org/10.1128/AEM.69.7.3965-3969.2003.

Geneva: WHO, 2020. Measles and rubella strategic framework 2021–2030.

Gentry, Z., Zhao, L., Faust, R.A., David, R.E., Norton, J., Xagoraraki, I., 2023. Wastewater surveillance beyond COVID-19: a ranking system for communicable disease testing in the tri-county Detroit area, Michigan, USA. Front. Public Health 11. https://doi.org/10.3389/fpubh.2023.1178515.

Group, T.dMIQE, Huggett, J.F., 2020. The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020. Clin. Chem. 66, 1012–1029. https://doi.org/10.1093/clinchem/hvaa125.

Guerra, F.M., Bolotin, S., Lim, G., Heffernan, J., Deeks, S.L., Li, Y., Crowcroft, N.S., 2017. The basic reproduction number (R0) of measles: a systematic review. The Lancet Infectious Diseases 17, e420–e428. https://doi.org/10.1016/S1473-3099(17)30307-9.

Hall, T., 2011. BioEdit: An important software for molecular biology. GERF Bulletin of Biosciences.

Hayes, E.K., Gouthro, M.T., LeBlanc, J.J., Gagnon, G.A., 2023. Simultaneous detection of SARS-CoV-2, influenza A, respiratory syncytial virus, and measles in wastewater by multiplex RT-qPCR. Sci. Total Environ. 889, 164261. https://doi.org/10.1016/j.scitotenv.2023.164261.

Hübschen, J.M., Gouandjika-Vasilache, I., Dina, J., 2022. Measles. Lancet 399, 678–690. https://doi.org/10.1016/S0140-6736(21)02004-3.

Hummel, K.B., Lowe, L., Bellini, W.J., Rota, P.A., 2006. Development of quantitative gene-specific real-time RT-PCR assays for the detection of measles virus in clinical specimens. J. Virol. Methods 132, 166–173. https://doi.org/10.1016/j.jviromet.2005.10.006.

Jourdain, F., Toro, L., Senta-Loÿs, Z., Deryene, M., Mokni, W., Azevedo Da Graça, T., Le Strat, Y., Rahali, S., Yamada, A., Maisa, A., Pretet, M., Sudour, J., Cordevant, C., Chesnot, T., Roman, V., Wilhelm, A., Gassilloud, B., Mouly, D., 2025. Wastewater-Based Epidemiological Surveillance in France: The SUM'EAU Network. Microorganisms 13. https://doi.org/10.3390/microorganisms13020281.

Kaic, B., Gjenero-Margan, I., Aleraj, B., Vilibić-Čavlek, T., Santak, M., Cvitković, A., Nemeth-Blazic, T., Ivic Hofman, I., 2010. Spotlight on measles 2010: Excretion of vaccine strain measles virus in urine and pharyngeal secretions of a child with vaccine associated febrile rash illness, Croatia, March 2010. Eurosurveillance 15. https://doi.org/10.2807/ese.15.35.19652-en.

Mahase, E., 2025. WHO warns "measles is back" as virus spreads across Europe, America, and Afghanistan. BMJ 388, r528. https://doi.org/10.1136/bmj.r528.

Moss, W.J., Griffin, D.E., 2006. Global measles elimination. Nat. Rev. Microbiol. 4, 900–908. https://doi.org/10.1038/nrmicro1550.

National Academies of Sciences, Engineering, and Medicine, 2023. Wastewater-based Disease Surveillance for Public Health Action. National Academies Press, (US), Washington (DC).

Ndlovu, N., Mabasa, V., Sankar, C., Msomi, N., Phalane, E., Singh, N., Gwala, S., Els, F., Macheke, M., Maposa, S., Yousif, M., McCarthy, K.M., 2024. Wastewater testing during the South African 2022-2023 measles outbreak demonstrates the potential of environmental surveillance to support measles elimination. medRxiv. https://doi.org/10.1101/2024.09.01.24312904.

Price, M., Simpson, B.S., Tscharke, B.J., Ahmed, F., Keller, E.L., Sussex, H., Kah, M., Sila-Nowicka, K., Chappell, A., Gerber, C., Trowsdale, S., 2024. Reporting population size in wastewater-based epidemiology: A scoping review. Sci. Total Environ. 953, 176076. https://doi.org/10.1016/j.scitotenv.2024.176076.

Rački, N., Dreo, T., Gutierrez-Aguirre, I., Blejec, A., Ravnikar, M., 2014. Reverse transcriptase droplet digital PCR shows high resilience to PCR inhibitors from plant, soil and water samples. Plant Methods 10, 42. https://doi.org/10.1186/s13007-014-0042-6.

Rector, A., Bloemen, M., Hoorelbeke, B., Van Ranst, M., Wollants, E., 2025. Detection of Measles Virus Genotype D8 in Wastewater of the Brussels Capital Region, Belgium. J. Med. Virol. 97, e70251. https://doi.org/10.1002/jmv.70251.

Rota, P.A., Khan, A.S., Durigon, E., Yuran, T., Villamarzo, Y.S., Bellini, W.J., 1995. Detection of measles virus RNA in urine specimens from vaccine recipients. J. Clinical Microbiol. 33, 2485–2488. https://doi.org/10.1128/jcm.33.9.2485-2488.1995.

Santé Publique France, 2025. La rougeole en France, bilan annuel 2024.

Shaw, A.G., Troman, C., Akello, J.O., O'Reilly, K.M., Gauld, J., Grow, S., Grassly, N., Steele, D., Blazes, D., Kumar, S., Environmental Surveillance Working Group, 2023. Defining a research agenda for environmental wastewater surveillance of pathogens. Nat. Med. 29, 2155–2157. https://doi.org/10.1038/s41591-023-02457-7.

The European Commission, 2021. Commission Recommendation (EU) 2021/472 of 17 March 2021 on a common approach to establish a systematic surveillance of SARS-CoV-2 and its variants in wastewaters in the EU.

The jamovi project, 2024. [Computer Software]. Retrieved from. https://www.jamovi.org.

Toro, L., de Valk, H., Zanetti, L., Huot, C., Tarantola, A., Fournet, N., Moulin, L., Atoui, A., Gassilloud, B., Mouly, D., Jourdain, F., 2024. Pathogen prioritisation for wastewater surveillance ahead of the Paris 2024 Olympic and Paralympic Games, France. Euro Surveill 29, 2400231. https://doi.org/10.2807/1560-7917.ES.2024.29.28.2400231.

UNICEF, WHO/Europe, 2025. European Region reports highest number of measles cases in more than 25 years.

Untergasser, A., Cutcutache, I., Koressaar, T., Ye, J., Faircloth, B.C., Remm, M., Rozen, S. G., 2012. Primer3–new capabilities and interfaces. Nucleic Acids Res. 40, e115. https://doi.org/10.1093/nar/gks596.

Wade, M.J., Jacomo, A.L., Armenise, E., Brown, M.R., Bunce, J.T., Cameron, G.J., Fang, Z., Farkas, K., Gilpin, D.F., Graham, D.W., Grimsley, J.M.S., Hart, A., Hoffmann, T., Jackson, K.J., Jones, D.L., Lilley, C.J., McGrath, J.W., McKinley, J.M., McSparron, C., Nejad, B.F., Morvan, M., Quintela-Baluja, M., Roberts, A.M.I., Singer, A.C., Souque, C., Speight, V.L., Sweetapple, C., Walker, D., Watts, G., Weightman, A., Kasprzyk-Hordern, B., 2022. Understanding and managing uncertainty and variability for wastewater monitoring beyond the pandemic: Lessons learned from the United Kingdom national COVID-19 surveillance programmes. J. Hazardous Mater. 424, 127456. https://doi.org/10.1016/j.jhazmat.2021.127456.

Wang, R., Hozumi, Y., Yin, C., Wei, G.-W., 2020. Mutations on COVID-19 diagnostic targets. Genomics 112, 5204–5213. https://doi.org/10.1016/j.ygeno.2020.09.028.

WHO, 2024. Wastewater and environmental surveillance for one or more pathogens: Guidance on prioritization, implementation and integration (No. Pilot version 6 Dec 2024).

WHO, 2020. World Health Organization Immunization Repository.

Wolf, S., Hewitt, J., Greening, G.E., 2010. Viral multiplex quantitative PCR assays for tracking sources of fecal contamination. Appl. Environ. Microbiol. 76, 1388–1394. https://doi.org/10.1128/AEM.02249-09.

Wu, J., Wang, M.X., Kalvapalle, P., Nute, M., Treangen, T.J., Ensor, K., Hopkins, L., Poretsky, R., Stadler, L.B., 2024. Multiplexed Detection, Partitioning, and Persistence of Wild-Type and Vaccine Strains of Measles, Mumps, and Rubella Viruses in Wastewater. Environ. Sci. Technol. 58, 21930–21941. https://doi.org/10.1021/acs.est.4c05344.

Ziegler, K., Steininger, P., Ziegler, R., Steinmann, J., Korn, K., Ensser, A., 2020. SARS-CoV-2 samples may escape detection because of a single point mutation in the N gene. Eurosurveillance. https://doi.org/10.2807/1560-7917.ES.2020.25.39.2001650.