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Wastewater-based surveillance for measles, mumps and rubella in Switzerland

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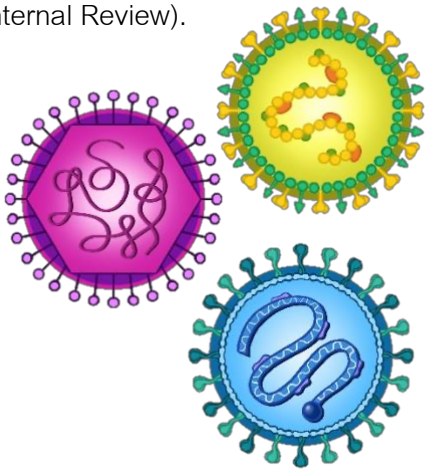

Summary

Measles, mumps, and rubella are highly contagious viral diseases that can cause severe health complications, particularly in children and the elderly. To reduce risks, vaccination with the Measles, Mumps, Rubella (MMR) vaccine is recommended, however outbreaks can still occur in communities, particularly those with lagging vaccination coverage. A recent example occurred from January to March 2024 in Lausanne when a measles outbreak emerged involving 51 identified cases. To help support Switzerland's strategy to monitor and track these three pathogens, we have developed two duplex digital PCR (dPCR) assays for wastewater screening based on previously published assays. The assays targeting: i) measles wild type (WT) and measles vaccine (VA) strains, and ii) mumps and rubella. In this report, we describe the development and validation of the two dPCR assays. The measles WT/VA assay was further applied retrospectively on wastewater extracts collected during the 2024 outbreak in Lausanne. Our findings showed that wastewater viral loads were temporally associated with clinical case data. The developed assays can be implemented to complement clinical-based surveillance, supporting a rapid response to outbreaks, tracking of relative changes, and helping to inform mitigation strategies.

Background

Measles is a severe respiratory disease that is very contagious and can lead to complications such as pneumonia, encephalitis, and, in some cases, death[1]. Measles is caused by the measles virus, an enveloped virus with a single-stranded negative-sense RNA genome. The virus belongs to the *Morbillivirus* genus in the *Paramyxoviridae* family, with only one serotype identified so far. Protection against measles is primarily achieved through the Measles, Mumps, Rubella (MMR) vaccine, which elicits immune responses against all three viruses. However, vaccination efficacy is not universal. Due to a combination of incomplete protection and contagiousness, measles requires >95% vaccination coverage to achieve herd immunity[2]. Indeed, although measles two-dose vaccine coverage in Switzerland is high (91% for 2 year olds, 94% for 8 year olds, and 96% for 16 year olds in 2023[3]), there was a recent outbreak in the canton of Vaud, Switzerland, from January to March 2024, which involved 51 confirmed cases[4]. The outbreak was initiated by an imported case, and 37 (72.5%) of the infected people had previously been vaccinated. Investigations revealed that even vaccinated people were symptomatic and capable of spreading the virus. This emphasizes the need for proactive surveillance, regardless of high vaccination coverage.

Mumps and rubella are included in the same vaccine as measles due to their potential to cause serious public health issues. Mumps, also caused by a virus from the *Paramyxoviridae* family, can lead to complications such as orchitis, meningitis, and hearing loss[5]. Despite vaccination, outbreaks still occur, often in populations where immunity has waned[6]. Rubella, caused by the rubella virus from the *Togaviridae* family, is of particular concern for pregnant women, as infection can result in congenital rubella syndrome (CRS), which can cause severe birth defects or miscarriage[7].

Given the public health risks associated with these pathogens, their inclusion in a combined vaccine simplifies immunization schedules. MMR vaccination rates as of 2023 have experienced a slight decline (-3%) compared to the previous years, interrupting the prior 20-year trend of consistent year-over-year growth for 2 year olds (all genders)[3]. Vaccination rate has stagnated at 94% from the year 2017 to 2023 for 8 year olds (all genders) and at 96% from the year 2020 to 2023 for 16 year olds (all genders)[3]. As part of Switzerland's efforts to track and monitor these pathogens, we have developed two digital PCR (dPCR) assays that can detect RNA in wastewater from: i) wild type and vaccine strains of measles virus, differentiated, and ii) all strains of mumps and rubella viruses, non-differentiated. These tools enhance environmental surveillance, allowing rapid scaling up of monitoring during outbreaks, such as the one in Lausanne, and providing a timely response when positive cases are reported from clinics in the future.

Material and Methods

Experimental design

We have developed two distinct duplex digital PCR (dPCR) assays for targeting: i) measles wild type (WT) strains and measles vaccine strains (VA), and ii) mumps and rubella (all strains for both targets). Both assays were adapted from Wu and colleagues[8]. Assays were first validated in single-plex assays and then validated as duplex assays.

To evaluate the assays' linearity, three serial dilutions of positive material (synthetic DNA for measles WT and extracted viral RNA from vaccine for measles VA, mumps and rubella) were measured in technical triplicates. Duplex assays were developed by merging individual single-plex assays. For multiplex validation, positive material quantification was assessed by both single-plex and duplex assays in parallel. Limit of Detection (LoD) and Quantification (LoQ) were identified by measuring decreasing concentrations with dPCR and by model fitting[9].

Following a measles outbreak in Lausanne, the Measles WT/VA assay was retrospectively applied to wastewater extracts collected over three months (January to March 2024) from the STEP Vidy wastewater treatment plant (WWTP), which serves the entire catchment area of Lausanne. Potential PCR inhibition in wastewater extracts was measured using spike-in experiments with synthetic DNA target measles B3 (gBlock®, Integrated DNA Technologies [IDT], USA) for a subset of samples tested.

MMR positive controls, primers, and probes

The two duplex assays were optimized using two types of positive controls: i) live-vaccine strains for measles, mumps, and rubella (Cat No. M0210000, M3600000, and R2000000), provided by European Directorate for the Quality of Medicines & HealthCare extracted with the QiaAmp Viral RNA MiniKit (Qiagen, Germany) following manufacturer instructions, and ii) a synthetic DNA construct for the most recent circulating genotype of measles (i.e., B3 genotype) (gBlock®, IDT, USA]) (Supplemental Table S1).

Primers and probe sequences were taken directly from Wu et al.[8] and were manufactured at IDT (USA). Fluorophore for the measles WT probe was modified to contain a Freedom Cy®5.5 dye and an Iowa Black® RQ Dark Quencher. Fluorophore for the measles VA probe was modified to contain an Iowa Black® RQ Dark Quencher (Supplemental Table S2).

Digital PCR assays

The two duplexes were developed (measles WT/VA, mumps and rubella) on the Naica® system 6-color digital PCR system (Stilla Technologies, France). PCR pre-reaction volume was 27 µl, containing 5.4 µl of sample template and 21.6 µl of mastermix components (Supplemental Table S3). Twenty-five µl of the total PCR reaction was pipetted into the chamber of the Sapphire chip (Stilla Technologies). The chip was then placed inside of a Geode (Stilla Technologies) for thermocycling and partitioning with the following steps: partitioning for 12 min at 40°C, reverse transcription at 50°C for 1 hour, enzyme activation at 95°C for 10 minutes, then 40 cycles of denaturation at 94°C for 30 seconds and annealing at 55°C for 1 minute, then finally an end cycle pressure release was added according to manufacturer's instructions.

Comparison of single-plex to duplex assays performance

To ensure that primer and probe interactions were minimal, quantification of positive material was compared between single-plex and duplex for both duplex assays. To accomplish this, the same positive control – mix of measles B3 strain synthetic DNA and measles vaccine strain for the measles WT/VA duplex, mix of mumps vaccine and rubella vaccine strain for the mumps/rubella duplex – was 10-fold diluted three times, and then measured in single-plex and duplex for both duplexes in technical replicates. The concentrations of individual replicates were averaged, and paired t-tests were performed to determine whether significant difference existed between single-plex and duplex concentrations for each dilution. Significance was set to $p = 0.05$.

Assays' sensitivity: Limit of detection and Limit of quantification

The multistep approach used to determine the LoD and LoQ is the same methodology described in the 2024 report, "Wastewater-based poliovirus surveillance using digital PCR". Before LoD and LoQ were assessed, we defined a sample as positive (i.e., detected) if there were three or more positive partitions (i.e., droplets), which corresponds to approximately 5 genome copies per reaction[10]. LoD, representing an assessment of the sensitivity of our assay, was then found and defined as the concentration at which a sample would be detected with 95% confidence. The LoD is determined empirically using multiple replicates of dilutions at low concentrations, and then fitting a dose-response model adapted for qPCR as described by Klymus et al.[9], where fit conditions were set to "best" or the model with the lowest residual error. The LoQ, which is the concentration at which the quantitative estimate of a concentration is a reliable estimate, was defined here as concentration at which the coefficient of variation (CoV) was 30%. To determine the LoQ, multiple replicate dilutions at moderate-to-low concentrations were measured, with the resultant data fit to a power model, $CoV = a \cdot C_{dpcr}^{-k}$, where CoV is the coefficient of variation, a is the scaling factor, C_{dpcr} is the concentration measured by dPCR, and k is the power relationship exponent. Both LoD and LoQ models were fitted with experimental data generated by measuring a minimum of 20 replicates at decreasing concentrations ranging from 6.4 to 110 gc/reaction for measles wild type (B3 gBlock®), 8.4 to 40 gc/reaction for measles vaccine, 8.4 to 30 gc/reaction for mumps, and 7.7 to 28 gc/reaction for rubella.

Both LoD and LoQ values were first expressed in gc/reaction and then multiplied by 1'200 (reactions/liter), which is a conversion factor to units of gc/L wastewater. The conversion factor is specific to our nucleic acid extraction, dilution factor, and digital PCR detection protocols (Equation 1).

$$\left[\frac{gc}{L}\right] = \left[\frac{gc}{reaction}\right] * 3\ (dilution) * \frac{1}{5\frac{\mu L\ extract}{reaction}} * 80\ \mu L\ extracted * \frac{1}{40mL\ wastewater} * \frac{1000mL}{1L} \quad (1)$$

Implementation of the Measles WT/VA duplex assay on wastewater: a time series

Corresponding to the most recent measles outbreak in Lausanne city between January and March 2024, twenty-five 24-hr composite influent samples from STEP Vidy WWTP were measured for measles WT/VA between January 8th to March 23rd (Supplemental Table S4). All samples were extracted using a protocol described in a previous study[11], and extracts were stored at -80°C until analysis. The measured extract was three-fold diluted prior to dPCR analysis. Time series data was not generated for mumps and rubella, as no specific outbreak was reported.

PCR inhibition quality control

To quantify the inhibitory effects of the wastewater matrix on PCR amplification, synthetic DNA of Measles B3 (gBlock®, IDT, USA) was spiked in a random subset of the wastewater extracts used to generate the time series for Lausanne. PCR inhibition was not assessed for mumps and rubella since no time series was generated. Inhibition, expressed in percentage, was defined as one minus the ratio between the amount measured in a spiked extract divided by the amount spiked in plus the endogenous measles WT within the extract (Equation 2). Inhibition ranged from 0 to 100% with 0 indicating no inhibition, and 100% implying a completely inhibited sample.

$$Inhibition\ (\%) = \left(1 - \frac{Spiked\ wastewater\ extract\left[\frac{gc}{reaction}\right]}{Amount\ spiked\ in\left[\frac{gc}{reaction}\right] + Endogenous\ measles\ wt\left[\frac{gc}{reaction}\right]}\right) \quad (2)$$

Comparison between wastewater measles RNA load and clinical confirmed cases

In this report, data about clinical confirmed cases of measles was sourced from a previous study published by Cassini et al. (2024)[4]. Only qualitative observations were made between available case data and wastewater viral loads to describe temporal associations.

Results

Development and validation of a Measles WT/VA and a Mumps/Rubella duplex dPCR assay

Prior to multiplexing, we tested each target in single-plex assays to ensure the separation between positive and negative clusters, and we tested the linearity using synthetic positive controls. Each single-plex assay showed clear distinction between positive and negative clusters, and data was fitted to linear regression, leading to R^2 values equal or greater than 0.99 for each target (Supplemental Figure S1).

Preservation of target quantification from single-plex to duplex assays

Duplex assays were generated by merging individual single-plex assays. We compared the quantification performance of the duplex assays by running them in parallel to single-plex assays using the same positive controls at three distinct dilutions. We observed that concentrations did not significantly differ between single-plex and duplex for both measles WT/VA and mumps/rubella (Figure 1) ($p > 0.05$ for each pairwise comparison).

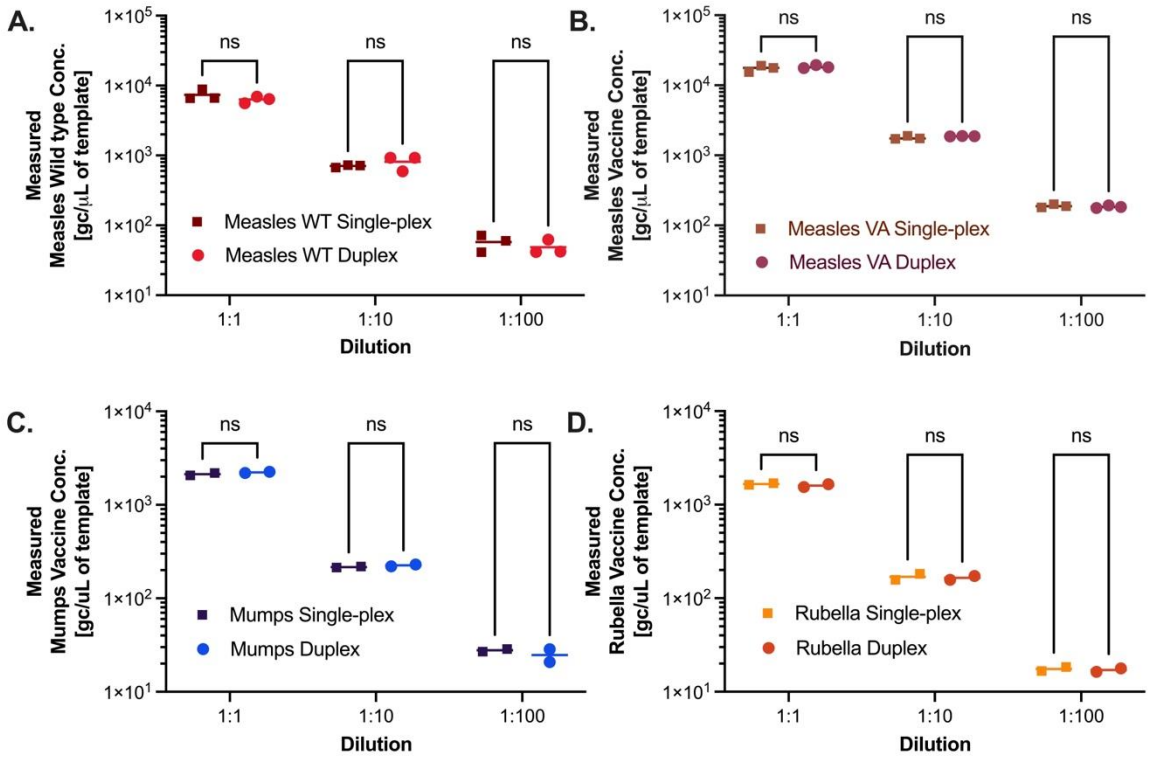

Figure 1. Quantification comparison between single-plex assays and duplexes using positive controls. X-axes show three 10-fold dilutions of the positive material. Y-axes indicate the measured concentrations using dPCR and expressed in genome copies (gc) per microliter (µl) of template. Within each dilution category, individual squares and circles represent replicate measurements of the same sample. Viral targets are specified with distinct colors and sorted by panel. Measles WT in panel A, measles vaccine in panel B, mumps vaccine in panel C, and rubella vaccine in panel D. Statistical significance was inferred using multiple paired t-tests (Supplemental Table S5). ns: not significant.

LoD and LoQ of duplex assays

We assessed the analytical sensitivity of each duplex assay and for each target by determining the LoD for multiple replicates (1, 2, 3, 4, 5, and 8-replicates) and LoQ (1-replicate) (Table 1). The selected models were fitted through detection probabilities of different concentrations for LoD (Supplemental Figure S2)and fitted through coefficients of variation of different concentrations for LoQ (Supplemental Figure S3).

Generally, routine wastewater monitoring as well as time series generated at Eawag are performed in technical duplicates. Therefore, here we use the 2-replicate LoD value for comparison. We observed that the 2-replicate LoD and LoQ values were similar for measles VA, mumps and rubella, with an LoD ranging from 9.2 to 9.8 gc/reaction and an LoQ ranging from 20.1 to 24.1 gc/reaction. However, for measles WT, 2-replicate LoD was approximately 1.8 times higher at 17.2 gc/reaction. LOQ was also higher for measles WT, approximately 5-7 times, at 128 gc/reaction.

Table 1. Replicate LoD and LoQ values for the two dPCR duplexes as determined in this study. Green text represents the unit gc/rxn: gene copies per reaction. Purple text represents the unit gc/L: gene copies per liter wastewater. rep: replicate.

		Rep. LoQ	Rep. LoD					
Virus Type	Unit	1	1	2	3	4	5	8
Measles Wild Type	[gc/rxn]	128	28.5	17.2	12.8	10.3	8.8	6.2
Measles Vaccine	[gc/rxn]	24.1	14	9.3	7.3	6.1	5.3	4
Mumps Vaccine	[gc/rxn]	23	15.3	9.2	6.9	5.6	4.7	3.4
Rubella Vaccine	[gc/rxn]	20.1	14.4	9.8	7.9	6.7	5.9	4.6
Measles Wild Type	[gc/L]	153'600	34'300	20'600	15'300	12'400	10'500	7'500
Measles Vaccine	[gc/L]	28'900	16'800	11'100	8'700	7'300	6'400	4'800
Mumps Vaccine	[gc/L]	27'600	18'300	11'100	8'300	6'700	5'700	4'100
Rubella Vaccine	[gc/L]	24'100	17'300	11'800	9'400	8'100	7'100	5'500

Measles WT/VA duplex dPCR assay: Lausanne outbreak in January to March 2024

From January to March 2024, 51 clinical cases of measles were confirmed in Lausanne[4]. We applied the Measles WT/VA duplex assay on wastewater samples from that period, which were collected at the treatment plant STEP Vidy. We tested a total of 25 wastewater extracts, and four extracts displayed more than three droplets when measuring with dPCR, which corresponds to our detection threshold. Notably, all quantified concentrations were below our modelled LoD, suggesting that although they were detected, the concentrations are low enough that our methods could not reliably detect them with 95% confidence. Interestingly, despite the low measured values, the analysis of measles RNA load in wastewater and confirmed case counts over a three-month period revealed a clear temporal association (Figure 2). Measles RNA load, expressed in genome copies per day per person (gc/d/person), showed a substantial increase from non-detect (i.e., equal or less than two droplets per reaction) in late January, and reached a first peak of $1.39 \times 10^7 \pm 8.06 \times 10^6$ gc/d/person in early February. This peak coincided with the highest recorded measles case count in one day (n = 7). A subsequent decrease in viral load was observed, followed by a second, larger peak in mid-February, which was not accompanied by an increase in confirmed cases. Following this second peak, both viral load and case counts declined substantially. By late February and early March, the viral load was not detectable. By mid- to late-March, there were no additional confirmed cases reported. Notably, measles vaccine strain was also measured, however, all samples were negative, despite catch-up vaccinations being offered by health practitioners in Lausanne on the 2nd of February 2024[4].

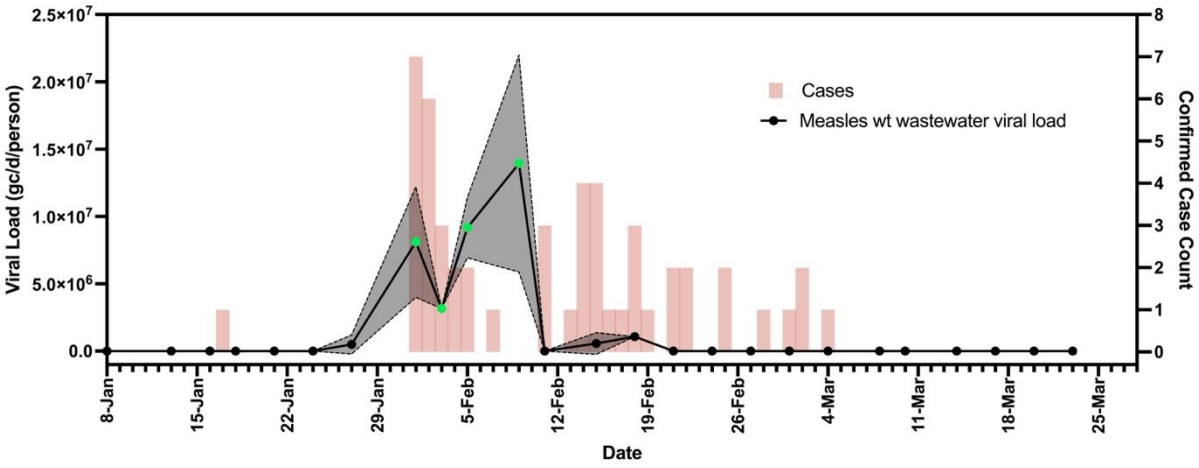

Figure 2 Temporal trends in measles viral load and confirmed case counts from early January to late March 2024. The primary y-axis (left) shows the viral load in wastewater (gc/d/person), represented by a black line with black (non-detect) and green (detect, i.e., least three droplets) data points, with the shaded region indicating the standard deviation around the mean of the viral load. The secondary y-axis (right) shows the number of confirmed measles cases, depicted as pink bars. Peaks in viral load occur in early and mid-February, coincide with the highest case counts, suggesting a correlation between wastewater viral load and infection prevalence. Viral load declines after mid-February and remains low through March, with no confirmed cases reported during this period.

A subset of the time series samples (10 out of 25) was measured for PCR inhibition and no substantial levels of inhibition were identified, as all values were below 9%, which is considerably below the acceptable threshold of 40% (Supplemental Table S6).

Main Takeaways and Conclusions

Development and validation of two duplex dPCR assays

We report the adaptation of two duplex dPCR assays, which target: i) measles WT/VA, and ii) mumps/rubella. For multiplexing validation, we addressed potential differences in quantification between single-plex and duplex assays, and we showed that the duplex assays performed as well as the single-plex assays. When focusing on the analytical sensitivity of measles vaccine and the mumps/rubella duplex assay, we identified an LoD of approximately 5 gc/reaction, which aligns with the threshold for signal detection (i.e., three droplets), and this observation was in line with a previous study[8]. However, the LoD reported for measles WT was higher, suggesting a lower sensitivity, which could be explained by the competitive nature of the assay. Specifically, the measles WT/VA assay is created with shared primers and two specific probes, where the probe targeting WT strains contains one mismatch with the synthetic DNA positive material, potentially leading to suboptimal amplification and higher variability. Despite the relatively high LoD value, we note lower concentrations remain detectable but with lower reliability. Therefore, we consider the assay specific, with capability of providing relative trends in viral loads.

Outbreak in Lausanne: application of Measles WT/VA dPCR assay to wastewater samples

The measles outbreak in Lausanne originated from a first imported case, which was confirmed on the 17th of January 2024. A previous study showed that 37 out of 51 total cases were linked to this first case[4]. The retrospective application of our assay to wastewater extracts from the same period successfully displayed positive signal for measles RNA, emphasizing the sensitivity of the assay despite the modest number of cases. We observed an increase in wastewater loads between the first confirmed case and the peak of cases (i.e., seven cases in a single day). There are two potential reasons for this, but we note that wastewater alone cannot distinguish between them. For example: i) only one of the subsequent secondary cases shed a substantial viral load into wastewater over the period of two weeks coincident to case confirmation, or ii) multiple secondary cases shed into wastewater over the period of two weeks, with wastewater dynamics mirroring spread dynamics (i.e., transmission rather than individual sporadic cases).

In conclusion, we developed two duplex-assays which are ready to be implemented when needed:

- The Mumps/Rubella dPCR assay:
    a. Displayed a high analytical sensitivity when validated with positive controls.
    b. Its efficiency in the context of wastewater surveillance needs to be further assessed.
- The Measles WT/VA dPCR assay:
    a. Was applied to wastewater samples and viral load data showed a temporal association with measles case data. Therefore, we consider the Measles WT/VA assay as an effective tool for outbreak monitoring.
    b. Showed rather high LoD value for measles WT but detected positive signal when tested on wastewater extracts even with a contained number of cases. Therefore, the assay could also be applied in the context of routine surveillance.

Contributors

This report was prepared by Charles Gan, Melissa Pitton, Jolinda de Korne, Christoph Ort, Timothy R. Julian. A first draft was reviewed by Marco Gabrielli (Internal Review). For further information or clarification about the contents of the report, please contact covid.abwasser@eawag.ch.

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Data Availability

All data are available at https://doi.org/10.25678/000E26 (measles wild type and measles vaccine data) and https://doi.org/10.25678/000E15 (mumps and rubella data).

Author Statement

This report contains portions of data already published in a peer-reviewed journal article located at the DOI: https://doi.org/10.1021/acs.estlett.5c00244 and a preprint at the following DOI: https://doi.org/10.1101/2025.03.11.25322836.

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Supplemental Material

Supplemental Tables

Supplemental Table S1. Characteristics of measles WT/VA, mumps, and rubella positive controls used.

Positive Material Type	Sequence/Genbank #	Details
Measles Schwarz vaccine strain	Genbank # GCA_031113345.1	Whole Genome
Mumps Urabe AM-9 vaccine strain	Genbank # GCA_031155735.1 (Considered Genotype B)[1]	Whole Genome
Rubella RA 27/3 vaccine strain	Genbank # GCA_031162565.1 (Considered Genotype 1A)[2]	Whole Genome
Measles B3 strain gBlock®	GTCCCTGCCCCTAGGTGTTGGCAGATCCACAG CAAAACCCGAAGAACTCCTCAAGGAGGCCACT GAGCTTGACATAGTTGTTAGACGTACAGCAGGG CTCAATGAAAAACTGGTGTTCTACAACAACACTC CACTAACTCTCCTCACACCTTGGAGAAAGGTCC TGACAACAGGGAGTGTCTTCAACGCAAATCAAG TGTGCAATGCGGTTAATCTGATACCGCTGGATA CCCCGCAGAGGTTCCGTGTTGTTTATATGAGCA TCACCCGTCTTTCAGATAACGGGTATTACACTGT TCCTAGAAGAATGCTGGAATTCAGATCAGTCAAT GCAGTGGCCTTCAACCTGCTGGTGACCCTTAGG ATTG	Nucleotide 3656-4025

Supplemental Table S2. MMR primers and probes used in this study. WT: wild type. VA: vaccine. /5Cy55/: Freedom Cy®5.5 dye modification from IDT. /5Cy5/: Freedom Cy®5 dye from IDT. /3IAbRQSp/ - Iowa Black® RQ Dark Quencher from IDT. /56FAM/: Fluorescein fluorophore from IDT. /ZEN: ZEN® internal quencher from IDT. /3IABkFQ/: Iowa Black® FQ Dark Quencher from IDT. /56ROXN/: Carboxy-X-rhodamine fluorophore from IDT.

Target	Oligo Type	Sequence (5' to 3')	Reference
Measles	Forward Primer	AATGAAAAACTGGTGTTCTACAA	Wu et al. (2024)
	Reverse Primer	GGTGATGCTCATATAAACAACAC	Wu et al. (2024)
	Probe – WT	/5Cy55/TATCCAGCGGTATCAGATTAACTGCATTGCA/3IAbRQSp/	Wu et al. (2024)
	Probe – VA	/5Cy5/TATCGAGCG/TAO/GTATCAGATTAACCGCATTGCA/3IAbRQSp/	Wu et al. (2024)
Mumps	Forward Primer	CATCTGGGCCAATTATAACTAC	Wu et al. (2024)
	Reverse Primer	GATGTTTGTCTTTTCAATCTGAA	Wu et al. (2024)
	Probe	/56FAM/TATGTCACC/ZEN/TGAGGACAAATGTCAG/3IABkFQ/	Wu et al. (2024)
Rubella	Forward Primer	CAGATGCAGGTTAGTGATCA	Wu et al. (2024)
	Reverse Primer	GACGTGTAGGGCTTCTTTAG	Wu et al. (2024)
	Probe	/56ROXN/CCCGCCGCCATTGGATCG AG/3IAbRQSp/	Wu et al. (2024)

Supplemental Table S3. Reaction composition for dPCR assays. The volume is expressed in microliters (µl) and refers to a single dPCR reaction.

Reagent	Volume (µL)
qScript® XLT 1-step RT-PCR Tough Mix	13.5
Target 1 Forward Primer (50µM)	0.27
Target 1 Reverse Primer (50µM)	0.27
Target 1 Probe (20µM)	0.27
Target 2 Forward Primer (50µM)	0.27
Target 2 Reverse Primer (50µM)	0.27
Target 2 Probe (20µM)	0.27
RNase Free Water	6.48
Template	5.4

Supplemental Table S4. Measles wild type RNA load in wastewater in Lausanne. C: concentration. AVG: mean. MSLS: measles. STD: standard deviation. WT: wild type

	Flow Rate (m^3)	C_{AVG} MSLS WT [gc/L_{ww}]	C_{STD} MSLS WT [gc/L_{ww}]	Load of MSLS WT (gc/d/person)	STD of MSLS WT load (gc/d/person)
2024-01-08	101322	0	0	0	0
2024-01-13	91343	0	0	0	0
2024-01-16	106184	0	0	0	0
2024-01-18	371137	0	0	0	0
2024-01-21	117022	0	0	0	0
2024-01-24	121704	0	0	0	0
2024-01-27	98898	1.20E+03	1.70E+03	5.07E+05	7.16E+05
2024-02-01	92045	1.92E+04	9.76E+03	8.11E+06	4.12E+06
2024-02-03	86453	7.50E+03	4.24E+02	3.17E+06	1.79E+05
2024-02-05	87525	2.18E+04	5.30E+03	9.18E+06	2.24E+06
2024-02-09	92602	3.30E+04	1.91E+04	1.39E+07	8.06E+06
2024-02-11	96108	0	0	0	0
2024-02-15	82418	1.35E+03	1.91E+03	5.70E+05	8.06E+05
2024-02-18	78897	2.55E+03	2.12E+02	1.08E+06	8.96E+04
2024-02-21	82531	0	0	0	0
2024-02-24	111999	0	0	0	0
2024-02-27	106912	0	0	0	0
2024-03-01	92043	0	0	0	0
2024-03-04	84652	0	0	0	0
2024-03-08	84507	0	0	0	0
2024-03-10	86489	0	0	0	0
2024-03-14	89249	0	0	0	0
2024-03-17	119503	0	0	0	0
2024-03-20	92613	0	0	0	0
2024-03-23	91342	0	0	0	0

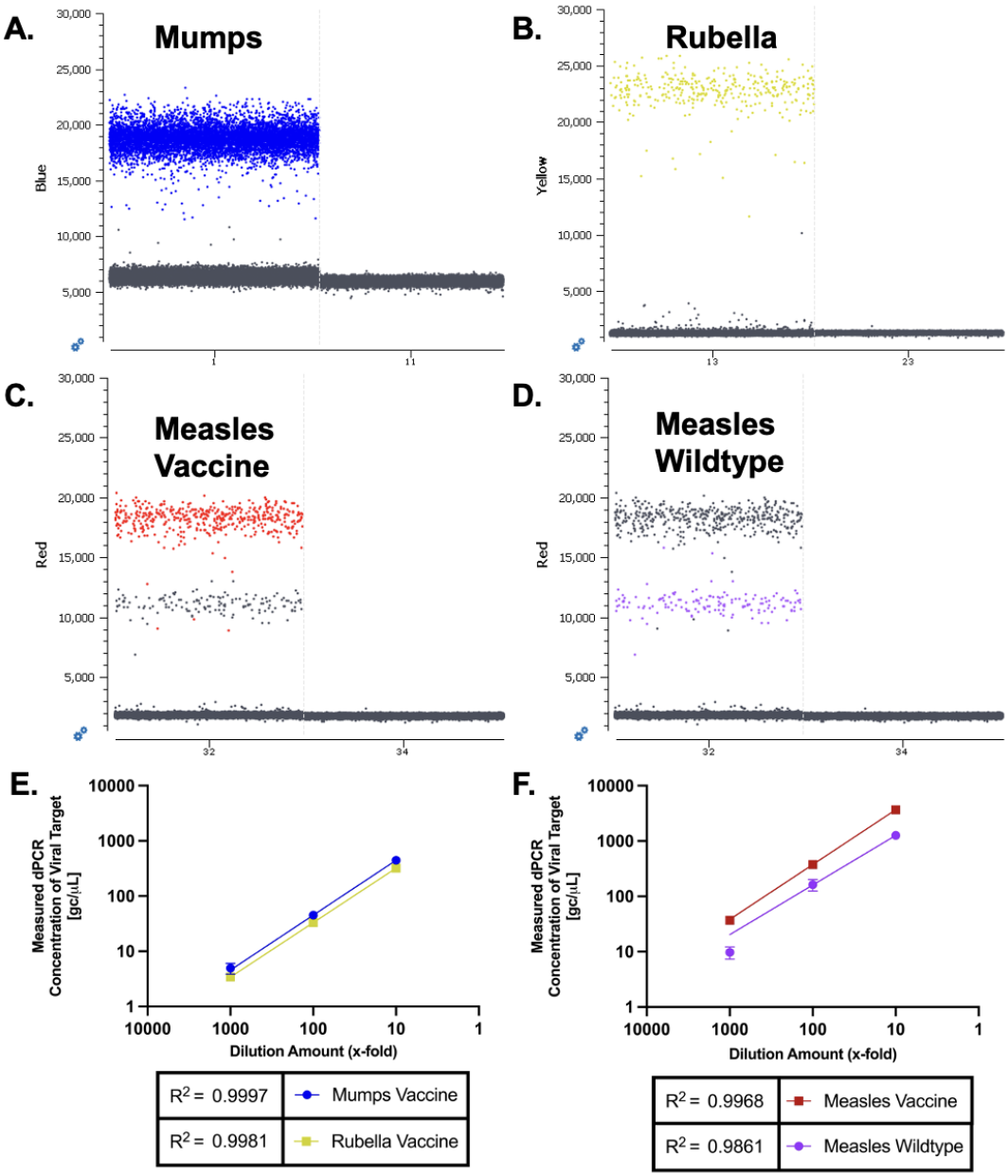
Supplemental Table S5. Comparison of quantification between single-plex and duplex dPCR assays: multiple paired t-tests. The table reports the *p* obtained for each pairwise comparison. All *p* values were above the significance threshold of 0.05.

Dilution factor	Measles wild type	Measles vaccine	Mumps	Rubella
1	0.31	0.25	0.22	0.60
10	0.46	0.26	0.46	0.85
100	0.49	0.48	0.49	0.83

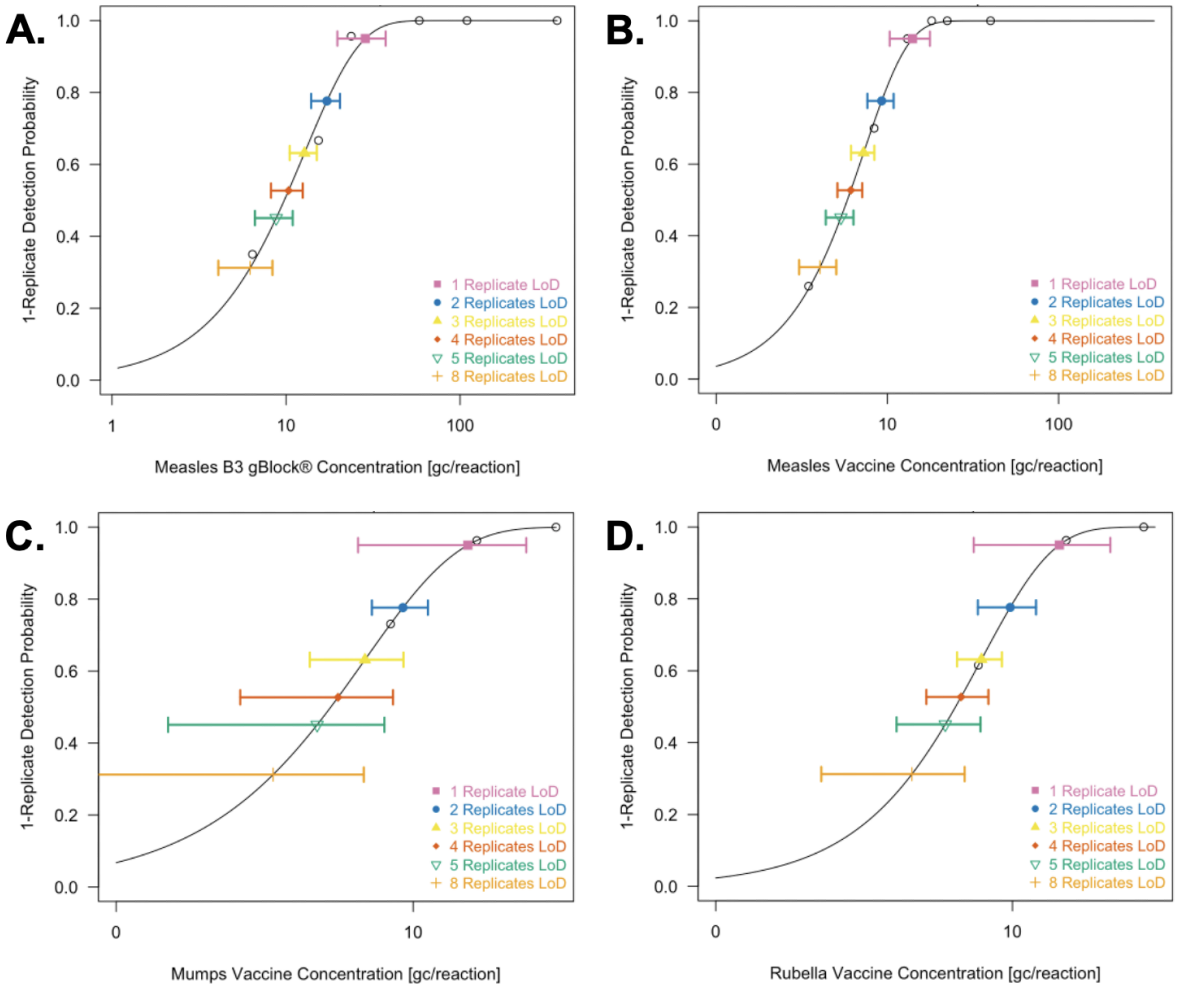
Supplemental Table S6. PCR inhibition detected on wastewater extracts used to generate the measles time series. C: concentration expressed in genome copies per reaction. AVG: mean. PCR inhibition was considered acceptable up to 40%.

Date	AVG of C_{spike}	AVG of C_{sample}	Inhibition (%)
2024-01-24	9961	9615	3
2024-01-27	9961	10348	-4
2024-02-01	9961	1923	3
2024-02-03	9961	2063	-4
2024-02-05	9961	1955	2
2024-02-09	9961	1827	8
2024-02-11	9961	2081	-4
2024-02-15	9961	2030	-2
2024-02-18	9961	2020	-1
2024-02-21	9961	1807	9

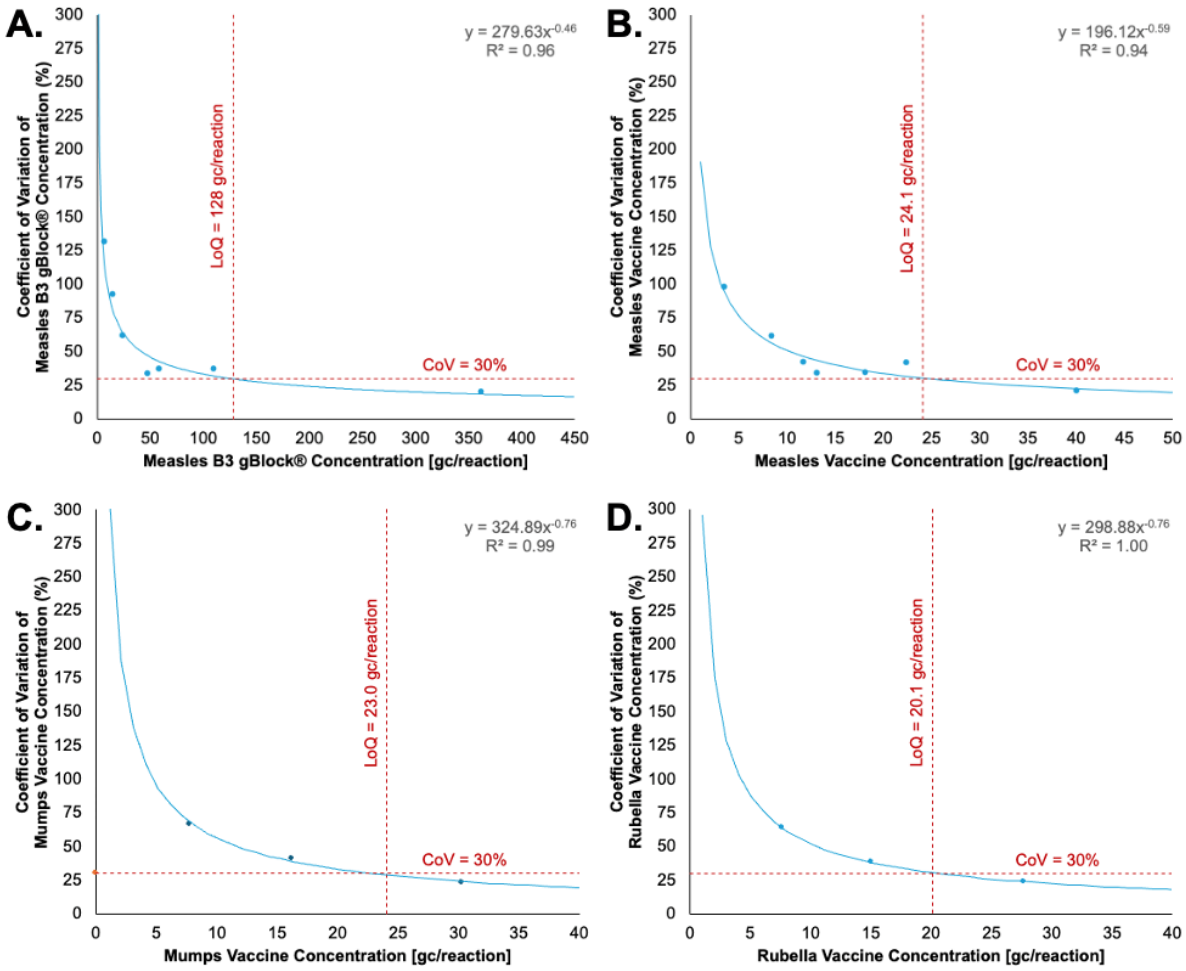
Supplemental Figures



Supplemental Figure S1. Validation of single-plex dPCR assays. (A), (B), (C), and (D): one dimensional representations of partition classification. X-axes show samples type (positive control on the left, and no-template control on the right). Each dot represents a single partition. Y-axes display the fluorescent intensity in the respective channels (labels indicate the color of the channel). (E) and (F): Linearity testing for single-plex assays. X-axes indicate the dilution factor (10-fold dilutions). Y-axes illustrate the measured concentration using dPCR and it is expressed in genome copies (gc) per microliter (µl) of template. Each dilution was measured in technical triplicates. Data was fitted to linear regression and R^2 values are displayed for each target.



Supplemental Figure S2. Modelled LoD of all targets measured using respective duplex assays. Weibull Type-2, two-parameter detection probability curves show the likelihood of detecting each viral target at increasing concentrations, expressed as genome copies (gc) per reaction. Panels A, B, C, and D represent different viral targets: (A) Measles B3 gBlock® concentration, (B) Measles vaccine concentration, (C) Mumps vaccine concentration and (D) Rubella vaccine concentration. Each colored line and symbol represent the limit of detection (LoD) for different numbers of replicates (1 to 8). The probability of detection approaches 1.0 as the target concentration increases, with LoD improving (shifted left) as replicate numbers increase. Error bars indicate the asymptotical Wald-type confidence intervals for each LoD threshold.



Supplemental Figure S3. Modelled LoQ of all targets measured using respective duplex assays. Viral target concentration is represented on the x-axis in gene copies per reaction. Coefficient of variation at each concentration is represented on the y-axis. A power law model is fitted through experimental data. The panels A, B, C and D represent the different viral targets. (A) Measles B3 gBlock® concentration: LoQ at 128 gc/reaction, with a model fit equation $y = 279.63x^{-0.46}$ and $R^2 = 0.96$ (B) Measles vaccine concentration: LoQ at 24.1 gc/reaction, with a model fit equation $y = 196.12x^{-0.59}$ and $R^2=0.94$ (C) Mumps vaccine concentration: LoQ at 23.0 gc/reaction, with a model fit equation $y=324.89x^{-0.76}$ and $R^2=0.99$. (D) Rubella vaccine concentration: LoQ at 20.1 gc/reaction, with a model fit equation $y=298.88x^{-0.76}$ and $R^2=1.00$. CoV decreases as target concentration increases. The dashed horizontal red line indicates the 30% CoV threshold for reliable quantification, and the vertical line represents the calculated LoQ for each target.

Supplemental References

1 Almansour, I. Mumps Vaccines: Current Challenges and Future Prospects. *Front Microbiol* **11**, 1999 (2020). https://doi.org/10.3389/fmicb.2020.01999

2 *Standardization of the nomenclature for genetic characteristics of wild-type rubella viruses*, <https://cdn.who.int/media/docs/default-source/immunization/vpd_surveillance/lab_networks/measles_rubella/rubella-nomemclature-report.pdf?sfvrsn=751e8270_3> (Accessed on: 12/11/2024).