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Wastewater surveillance predicts Mpox outbreak dynamics in a resource-limited urban setting

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ABSTRACT

Wastewater-based surveillance (WBS) has emerged as a scalable approach for monitoring emerging pathogens, yet its capacity to function as an early-warning system for Mpox virus (MPXV) in rapidly urbanizing sub-Saharan African settings remains unresolved.

We conducted weekly wastewater sampling at four facilities in the Kampala Metropolitan Area, Uganda, during the 2025 Mpox outbreak (February–September; $n = 128$ samples) and quantified MPXV DNA by real-time PCR. MPXV was detected at all sites (40.6–93.8% of weeks), demonstrating robust environmental detectability in a high-burden African city. However, temporal coupling between wastewater concentrations and clinical Mpox positivity was heterogeneous. After autocorrelation adjustment, concurrent correlations were generally weak and statistically underpowered. Lead–lag analyses revealed distinct site-dependent dynamics: the Bugolobi fecal sludge treatment facility exhibited a 3-week prospective signal ($r_s = 0.623$), whereas high-connectivity Nakivubo wastewater inlets 1 & 2 and Naalya stabilization pond displayed concurrent or lagged patterns. No association persisted after false discovery rate correction.

These findings show that WBS performance is not intrinsic to pathogen detection alone but emerges from the interaction between viral shedding, sewer network architecture, hydrological residence time, and health-seeking behaviour. Early-warning capacity is therefore a systems property of surveillance network design rather than a universal attribute of wastewater monitoring. Our results provide empirical evidence from sub-Saharan Africa that optimizing sewershed configuration and temporal resolution is essential before WBS can be operationalized as a predictive tool within national epidemic intelligence frameworks.

Keywords: Wastewater-based surveillance, monkeypox virus, Epidemic preparedness, Environmental Surveillance.

MAIN

Mpox is a zoonotic viral disease caused by the monkeypox virus (MPXV), an enveloped, double-stranded DNA virus belonging to the *Orthopoxvirus* genus of the *Poxviridae* family (1). The virus was first discovered in 1958 during research on monkeys in Denmark, and the first human case was documented in 1970 in the Democratic Republic of the Congo in a nine-month-old boy (2). Since then, Mpox has been documented predominantly in West and Central Africa. However, when more than 16,000 cases from 75 countries and territories, with 5 deaths, were reported, the World Health Organization (WHO) declared a Public Health Emergency of International Concern on July 23, 2022 (3). By August 2024, Mpox had been reported in more than 120 countries worldwide, resulting in over 100,000 laboratory-confirmed cases and more than 220 deaths globally (2).

In Africa, the outbreak has been particularly profound. From the beginning of 2024 to 26 August 2024, 13 African Union Member States reported a total of 22,863 Mpox cases (19,222 suspected and 3,641 confirmed) and 622 deaths, with an average case fatality rate (CFR) of 2.06% (95% CI: 0–4.26%) (4). In Uganda, the first two cases were confirmed in Kasese District on 24 July 2024, prompting the Ministry of Health to declare an outbreak on 2 August 2024 (5). As of 30 March 2025, Uganda had reported 4,881 confirmed cases across 103 districts, with a CFR of 0.78% (6).

Wastewater-based surveillance (WBS) has emerged as a cost-effective, real-time, and comprehensive approach for tracking infectious diseases. Unlike case-based clinical testing, it is not limited by symptom presence, access to healthcare, or diagnostic disparities. It monitors

pathogens excreted in urine, feces, respiratory secretions, and skin cells that eventually enter sewage networks, making raw wastewater an aggregated biological sample representing the entire community (7), including asymptomatic individuals and those who do not seek healthcare. Thus, WBS has been positioned as a crucial public health tool and has been successfully used to detect and monitor infectious diseases, such as polio and COVID-19, at the population level (7–9).

Recent studies confirm that individuals with Mpox shed viral DNA in stool, urine, saliva, and skin lesions, and that this DNA can enter wastewater via toilets, sinks, and shower drains (10,11). This biological shedding allows WBS to serve as a pooled community-level sample for tracking MPXV transmission. WBS has shown substantial value as an early warning tool for emerging disease outbreaks by detecting viral signals in sewage prior to, or in the absence of, clinical case reports (12). Moreover, the use of WBS in combination with genomic sequencing can further provide epidemiological insight into circulating lineages and their geospatial distribution, which are important for outbreak management (13).

Given the current outbreak dynamics and the limitations of routine testing, alternative surveillance strategies are urgently needed. The multiple shedding routes of MPXV, combined with the demonstrated success of WBS during the COVID-19 pandemic in universities, congregant living facilities, and urban communities, present a unique opportunity to apply this approach to Mpox surveillance (14,15).

This study compares WBS data with reported clinical cases to evaluate WBS's ability to detect early warning signs of Mpox spread. By correlating viral signals in sewage with clinical case data, we examine WBS's potential as an early warning system for Mpox transmission in high-risk populations, even when reported clinical case counts and case ascertainment are low.

RESULTS

Temporal characterization of MPXV wastewater concentrations across four treatment facilities

Weekly MPXV genomic copy concentrations were measured in wastewater influent at four treatment facilities in the Kampala Metropolitan Area over a 32-week surveillance period from February to September 2025. Detection frequencies varied substantially across facilities. Nakivubo Wastewater Plant Inlet 1 (Nakivubo WWTP Inlet 1) had the highest detection rate, with a detectable signal in 30 of 32 weeks (93.8%), followed by Bugolobi Fecal Sludge Treatment Plant (Bugolobi FSTP) (21/32, 65.6%), Naalya Wastewater Stabilization Pond (Naalya WWSP) (17/32, 53.1%), and Nakivubo Wastewater Plant Inlet 2 (Nakivubo WWTP Inlet 2) (13/32, 40.6%). Among detected weeks, log₁₀-transformed genomic copy concentrations per 100 mL (log₁₀ GC/100 mL) ranged from 2.74 to 6.85 across all facilities and weeks (*Figure 1*). Temporal dynamics were characterized by pronounced week-to-week variability at all sites, with episodic high-concentration events interspersed with non-detect weeks, particularly at Naalya WWSP and Nakivubo WWTP Inlet 2 (*Figure 1*).

Concurrent weekly clinical Mpox positivity in the Kampala Metropolitan Area remained elevated throughout the observation period, ranging from 11.1% to 100.0% (mean 61.8%, SD 20.4%, median 66.1%, IQR 51.3–75.5%), indicating sustained community transmission during the study period.

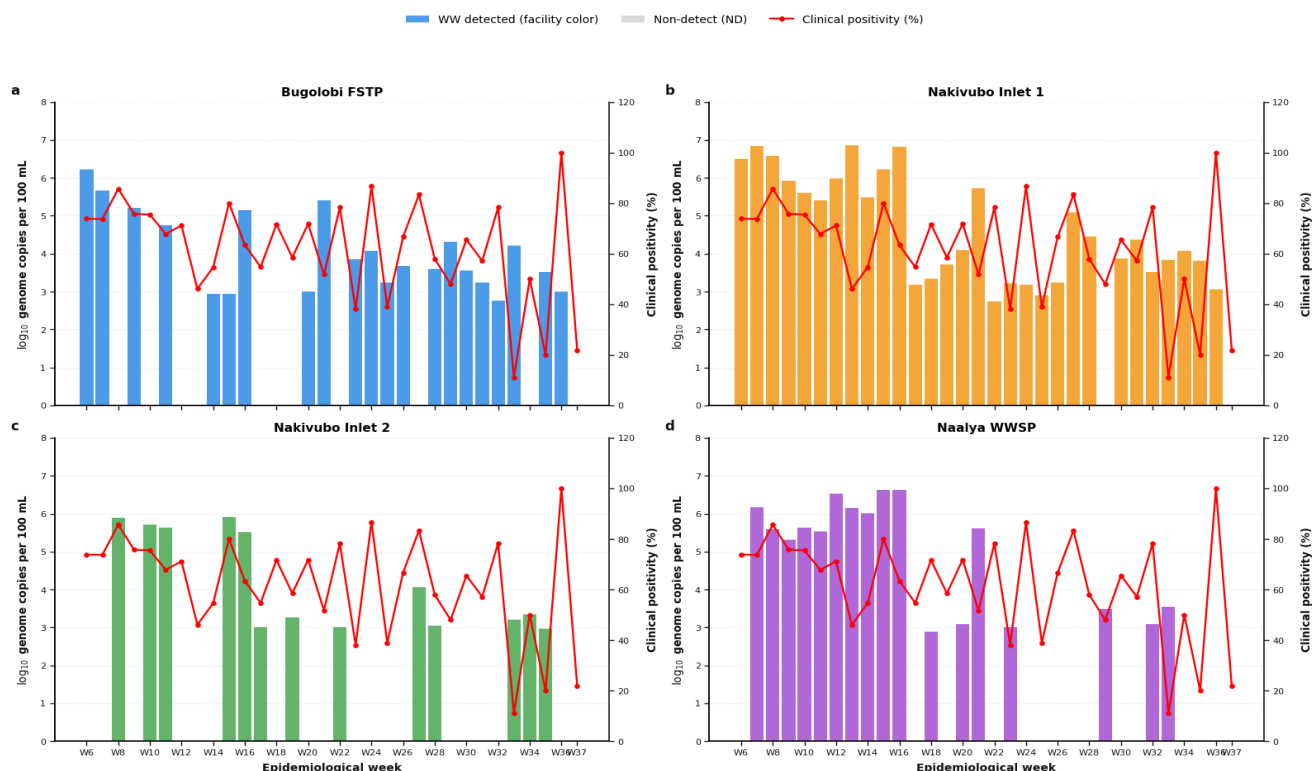


Figure 1: Weekly MPXV wastewater concentrations and clinical Mpox positivity in the Kampala Metropolitan Area, February–September 2025. Wastewater measurements from (a) Bugolobi FSTP, (b) Nakivubo WWTP Inlet 1, (c) Nakivubo WWTP Inlet 2, and (d) Naalya WWSP are shown as weekly $\log_{10}$ MPXV genome concentrations (genome copies per 100 mL). Grey bars indicate non-detect (ND) samples. Red lines represent weekly clinical Mpox positivity (% positive tests).

Median concentrations among detected weeks were highest at Naalya WWSP (5.60 $\log_{10}$ GC/100 mL; interquartile range [IQR] 3.48–6.16) and Nakivubo Inlet 1 (4.23; IQR 3.39–5.87), intermediate at Nakivubo WWTP Inlet 2 (3.35; IQR 3.05–5.63), and lowest at Bugolobi (3.68; IQR 3.24–4.75) (Table 2).

Table 1: Descriptive statistics for weekly $\log_{10}$ MPXV GC/100 mL at each facility (detected weeks only). ND, non-detect; IQR, interquartile range; GC, genomic copies.

Facility	n (detected)	ND weeks (%)	Median $\log_{10}$ GC/100 mL	IQR	Mean	SD	Range
Bugolobi FSTP	21	11 (34.4%)	3.678	3.239–4.748	4.016	1.019	2.763–6.221
Nakivubo WWTP Inlet 1	30	2 (6.2%)	4.230	3.387–5.866	4.654	1.378	2.739–6.853
Nakivubo WWTP Inlet 2	13	19 (59.4%)	3.347	3.046–5.627	4.200	1.296	2.974–5.916

Naalya WWSP	17	15 (46.9%)	5.600	3.480– 6.157	4.992	1.437	2.880– 6.627
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Distributional properties support non-parametric inference

Shapiro-Wilk tests of log₁₀ MPXV concentrations at detected weeks rejected the null hypothesis of normality at three of four facilities: Nakivubo WWTP Inlet 1 ($W = 0.905$, $p = 0.011$), Nakivubo WWTP Inlet 2 ($W = 0.766$, $p = 0.003$), and Naalya WSWP ($W = 0.830$, $p = 0.005$). Bugolobi FSTP returned a borderline non-significant result ($W = 0.916$, $p = 0.072$), consistent with approximate normality at this facility but insufficient to override the overall distributional evidence. Given non-normality at most sites, the bounded nature of the clinical positivity outcome, and the relatively small sample sizes typical of ecological surveillance time series, non-parametric estimators were selected as primary for all subsequent analyses.

MPXV concentrations differ significantly between Nakivubo WWTP inlet points

Pairwise Wilcoxon signed-rank tests, restricted to concurrently detected weeks and corrected for multiple comparisons using the Holm procedure, identified a single statistically significant difference in concentration among the six facility pairs examined. Nakivubo WWTP Inlet 1 had significantly higher MPXV concentrations than Nakivubo WWTP Inlet 2 in weeks when both were detectable ($W = 8.00$, $p_{raw} = 0.006$, $p_{Holm} = 0.037$; $n = 13$ paired weeks). No other facility pair differed significantly after correction (all $p_{Holm} > 0.26$; Supplementary file 1). The concentration difference between the two Nakivubo WWTP inlet points likely reflects distinct catchment characteristics or sampling positions relative to contributing flow volumes within the same treatment works, rather than absolute differences in community viral shedding.

Concurrent wastewater-clinical associations are constrained by autocorrelation and sample size.

Spearman rank correlations between weekly log₁₀ wastewater MPXV concentrations and same-week clinical positivity (lag 0) were estimated for each facility using only detected weeks. To contextualize inferential power, minimum detectable Spearman correlations (MDE) at 80% power (two-sided $\alpha = 0.05$) were computed before interpretation. MDE exceeded 0.60 at three of four facilities (Bugolobi FSTP: 0.58; Nakivubo WWTP Inlet 2: 0.71; Naalya WSP: 0.63), indicating that only strong associations were detectable given the observed effective sample sizes and that null results should not be interpreted as evidence of absent associations. Lag-1 autocorrelation was substantial in the wastewater series at Nakivubo WWTP Inlet 1 ($r_1 = 0.657$) and Nakivubo WWTP Inlet 2 ($r_1 = 0.765$), and moderate at Naalya WSP ($r_1 = 0.529$) and Bugolobi FSTP ($r_1 = 0.256$). The clinical positivity series showed mild negative autocorrelation ($r_1 = -0.339$). Effective sample sizes after Quenouille-Bartlett correction were substantially reduced at high-autocorrelation facilities, most notably at Nakivubo WWTP Inlet 1 ($n_{eff} = 6.2$ from nominal $n = 30$) and Nakivubo WWTP Inlet 2 ($n_{eff} = 3.0$ from $n = 13$).

At lag 0, no facility returned a statistically significant Spearman correlation with clinical positivity after autocorrelation adjustment (all $p_{adj} > 0.53$; Figure 2). Nominally, Nakivubo WWTP Inlet 2 showed the strongest concurrent association ($r_s = 0.670$, 95% CI [0.19, 0.89], $p_{nominal} = 0.012$, $n = 13$). However, this result became non-significant after Quenouille-Bartlett correction ($p_{adj} = 0.532$), reflecting the very small autocorrelation-adjusted effective sample size at that facility. Correlations at Bugolobi FSTP ($r_s = -0.077$, $p = 0.741$), Nakivubo WWTP Inlet 1 ($r_s = 0.079$, $p = 0.677$), and Naalya WWSP ($r_s = 0.147$, $p = 0.573$) were weak and non-significant (Figure 2).

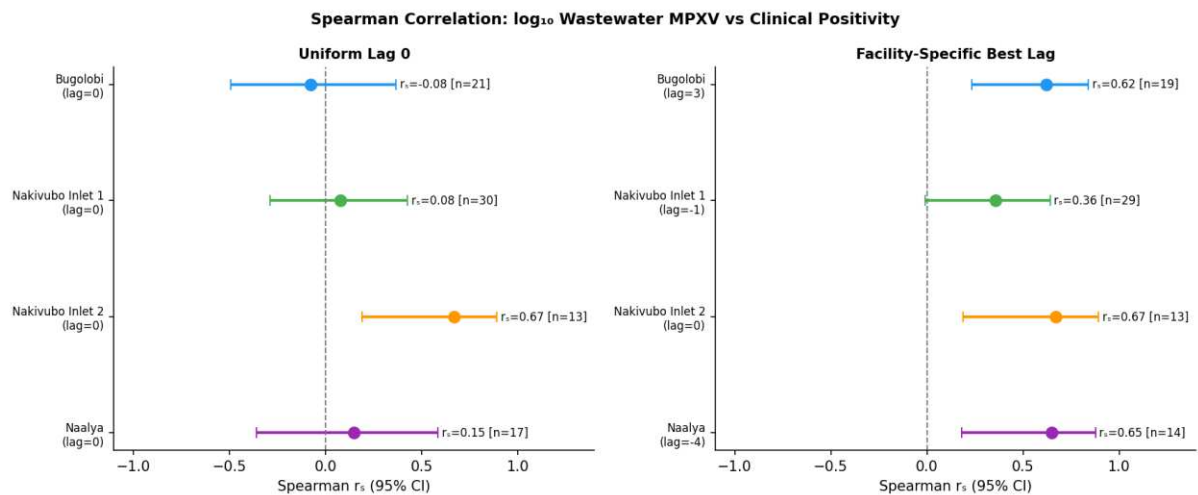


Figure 2: Fig. 4: Forest plots of Spearman r_s (95% Fisher Z-transformed confidence intervals) for each facility at uniform lag 0 (left) and facility-specific best lags (right). Dashed vertical lines indicate $r_s = 0$. Effective n per facility-lag pair is annotated

Exploratory lead-lag analysis reveals a prospective early-warning signal at Bugolobi FSTP

To evaluate the potential of WBS to anticipate clinical case detection, Spearman correlations were computed within the ± 4 -week lag window for each facility, with false discovery rate (FDR) control using the Benjamini–Hochberg (BH) procedure across all 36 facility-lag combinations. No individual facility-lag combination remained significant after the BH correction (all $p_{BH} > 0.05$), consistent with the exploratory nature of this analysis and the limited power of short ecological time series (Figure 3; Supplementary file). Interpretation, therefore, emphasized effect size, confidence interval width, and directional biological plausibility. The most notable finding was at Bugolobi FSTP, where a 3-week lag in wastewater relative to clinical positivity yielded the largest absolute Spearman correlation observed across any facility-lag combination ($r_s = 0.623$, 95% CI [0.24, 0.84], $p_{raw} = 0.004$, $p_{BH} = 0.146$, $n = 19$; Figure 3). This suggests that elevated MPXV wastewater concentrations at this facility precede an increase in clinical positivity by approximately 3 weeks, consistent with the combined latency of viral shedding onset, wastewater transit time, and healthcare-seeking and reporting delays. At Nakivubo WWTP Inlet 2, the strongest association remained at lag 0 ($r_s = 0.670$, $p_{BH} = 0.146$), suggesting that wastewater concentrations at this facility co-vary with clinical positivity in the same epidemiological week rather than providing advance signal. At Naalya WSP, the peak correlation was observed at lag -4 ($r_s = 0.649$, 95% CI [0.18, 0.88], $p_{raw} = 0.012$, $p_{BH} = 0.146$, $n = 14$), with clinical positivity appearing to precede the wastewater signal by 4 weeks. This pattern may reflect reporting lags in the clinical surveillance network, upstream subcatchment dynamics not captured at the inlet sampling point, or residual confounding from the small, spatially restricted paired sample. Nakivubo WWTP Inlet 1 showed a modest association at lag -1 ($r_s = 0.358$, 95% CI [−0.01, 0.64], $p_{raw} = 0.056$), which did not meet the screening threshold ($p < 0.20$) for designation as a prospective lead candidate (Figure 3).

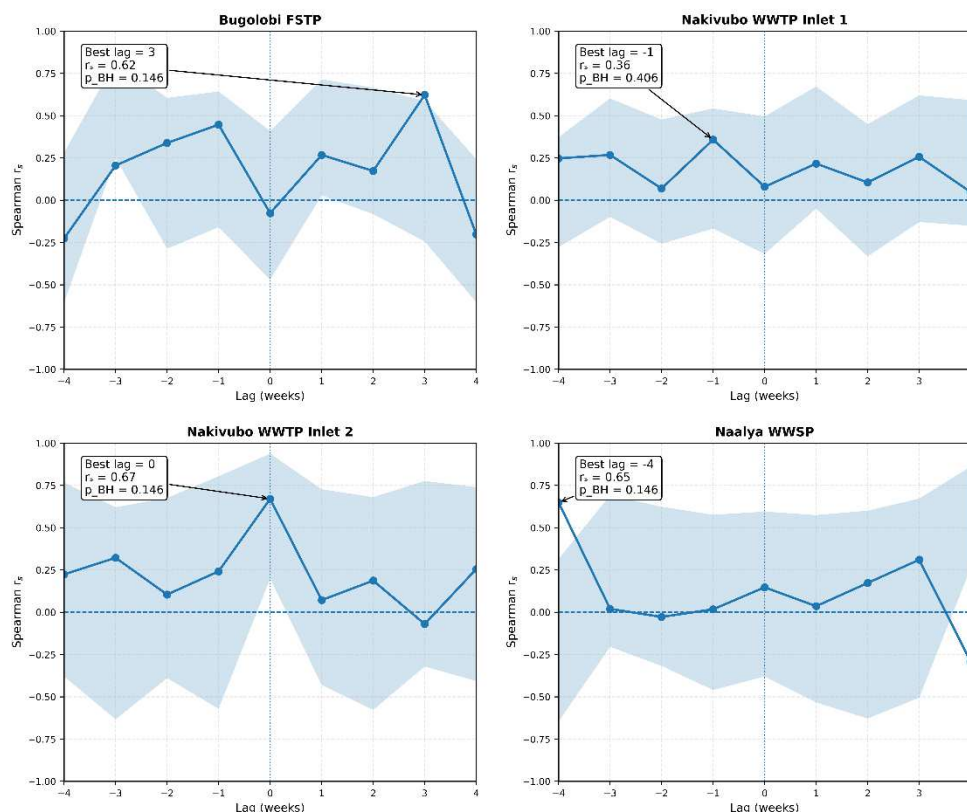


Figure 3: Lead-lag Spearman correlation across the four wastewater treatment facilities. Each cell shows the Spearman r_s between $\log_{10}$ MPXV GC/100 mL and weekly clinical Mpox positivity at lags from -4 to $+4$ weeks (positive values indicate wastewater

Wastewater MPXV concentration is a positive predictor of clinical positivity at facility-specific lags

At lag 0, only Nakivubo WWTP Inlet 2 showed a Theil-Sen slope whose bootstrap confidence interval excluded zero (slope = 10.54 percentage points per $\log_{10}$ unit (pp/ $\log_{10}$ unit), 95% CI [2.56, 24.84]; OLS: slope = 10.33, $p = 0.037$, $R^2 = 0.34$). No other facility returned a lag-0 slope CI excluding zero, consistent with the absence of concurrent correlations described above. At facility-specific best lags, the positive directional consistency across all four facilities was striking. Bugolobi FSTP at lag +3 yielded a Theil-Sen slope of 11.70 pp/ $\log_{10}$ unit (95% CI [4.83, 26.24]; OLS: slope = 11.36, $p = 0.018$, $R^2 = 0.29$), indicating that each order-of-magnitude increase in wastewater MPXV concentration was associated with approximately 12 pp higher clinical positivity three weeks later. Naalya WSP at lag -4 produced the largest OLS R^2 ($R^2 = 0.54$, $p = 0.003$), with a Theil-Sen slope of 5.57 (95% CI [2.58, 9.20]; OLS: 6.06), suggesting a robust quantitative relationship between Naalya WWSP wastewater concentrations and antecedent clinical positivity at this temporal displacement, though its mechanistic interpretation requires caution given the negative lag. Nakivubo WWTP Inlet 1 at lag -1 produced a Theil-Sen slope of 5.02 (95% CI $[-0.58, 10.04]$; OLS: 5.46, $p = 0.033$, $R^2 = 0.16$). Across all four facility-specific lag models, OLS slopes were directionally concordant with Theil-Sen estimates, supporting the robustness of the positive associations to the choice of estimator (Figure 4).

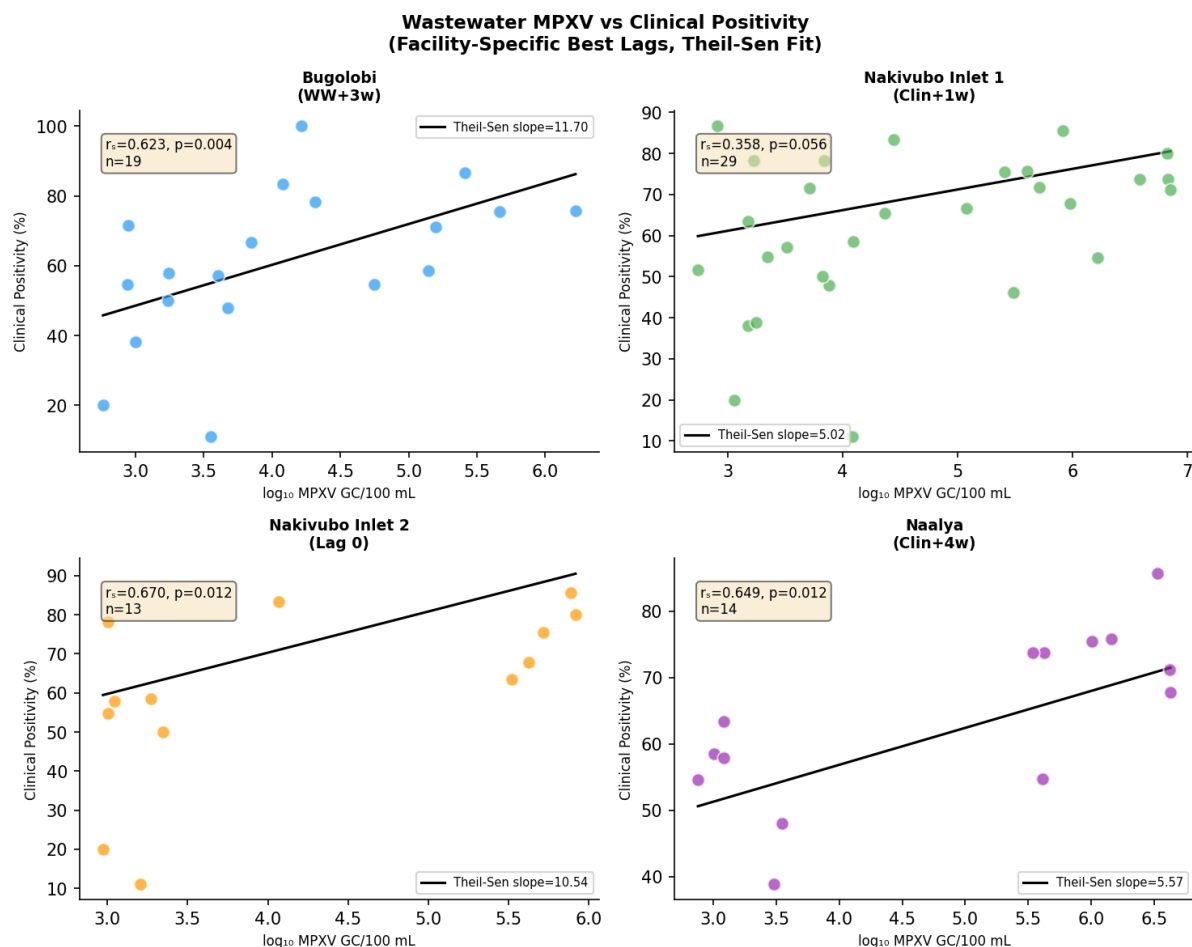


Figure 4: Scatter plots of $\log_{10}$ MPXV GC/100 mL versus clinical Mpox positivity at facility-specific best lags, with Theil-Sen regression fits (black lines).

DISCUSSION

This study provides the first evidence that WBS can detect the MPXV genomic signal in citywide wastewater across multiple treatment facilities in the Kampala Metropolitan Area, Uganda, and documents exploratory associations between wastewater MPXV concentrations and concurrent clinical Mpox positivity over a 32-week surveillance window from February to September 2025. Our findings situate Kampala among a small but growing cohort of cities globally in which MPXV DNA has been recovered from wastewater (16–18), and, critically, constitute the first such surveillance data from sub-Saharan Africa (SSA) during the second WHO-declared Public Health Emergency of International Concern (PHEIC) across Central and East Africa (19–21). Taken together, the results demonstrate both the feasibility and the methodological challenges of deploying WBS as an early-warning tool for emerging and re-emerging pathogens in resource-limited urban settings.

The most consequential finding was the 3-week lead of the Bugolobi FSTP wastewater signal over clinical positivity ($r_s = 0.623$; Theil-Sen slope: 11.70 pp/ $\log_{10}$ unit; 95% CI [4.83, 26.24]). A prospective lead of this duration is biologically plausible: MPXV rectal and anal shedding, the most likely route of wastewater entry given the predominantly sexual transmission network driving the 2024–2025 East African outbreak (22–25) begins in the prodromal phase and persists throughout the vesiculopustular rash stage for 2 to 4 weeks (24,26,27). This shedding window precedes or coincides with peak healthcare-seeking behavior, which, in low-resource

settings, is itself delayed by stigma, symptom misattribution, and limited diagnostic capacity (28,29). A 3-week wastewater lead is therefore consistent with the combined effects of pre-symptomatic shedding, delayed care-seeking, and the administrative lag inherent in passive clinical surveillance systems in the Kampala Metropolitan Area during this period.

This lead interval compares favorably with those observed during the 2022 global clade IIb outbreak, where WBS studies in California (30), Canada (15), and several European cities (16,17,31) reported wastewater detection preceding or coinciding with clinical case notification by 0-14 days in settings with established clinical surveillance and higher per-capita testing rates. The longer lead time at Bugolobi FSTP may reflect structural features of clinical surveillance in Kampala, including limited testing access, healthcare-seeking barriers, and probable case undercounting, all of which are characteristic of MPXV epidemiology in SSA (19,28,29) and which increase the comparative advantage of wastewater as an unbiased, population-level indicator. Analogous dynamics have been well-characterized in the SARS-CoV-2 WBS literature, where wastewater signal consistently anticipated clinical reports by 4–7 days in high-surveillance settings (32–34) but demonstrated wider leads in settings where case-ascertainment rates were depressed (35,36).

The absence of a statistically detectable concurrent (lag-0) association at most facilities after autocorrelation adjustment does not contradict the lead-lag finding; rather, it reinforces the interpretation that wastewater concentrations encode epidemiological information at a temporal offset from reported clinical positivity, consistent with the expected biology of shedding and reporting delays. Importantly, this null lag-0 result mirrors observations from the Chicago mpox WBS programme, where wastewater signal and case counts diverged contemporaneously but aligned when appropriately lagged (18), and from Switzerland, where wastewater-clinical correlations were strongest at lags of 1–2 weeks (37).

Uganda reported its first confirmed cases of clade Ib MPXV infections in late 2024. Genomic and epidemiological data confirmed these were imported via cross-border transmission from the DRC (28). The clade Ib variant, a sub-lineage of clade I first characterized in the Kamituga mining district of South Kivu province (23), is distinguished from the clade IIb lineage responsible for the 2022 global outbreak by a higher case-fatality rate, a broader demographic distribution of cases that includes children, and, critically, efficient human-to-human transmission through both sexual and household contact (19,22,38). The evolving epidemiology of clade Ib across Central and East Africa, characterized by cross-border spread to Uganda, Rwanda, Burundi, and Kenya (19,21), makes sustained community-level surveillance in Kampala not merely locally relevant but also of regional public health significance.

The high and sustained clinical Mpox positivity observed throughout our study window (median 66.1%, IQR 51.3–75.5%) is consistent with the epidemiological trajectory described by Ndembu et al. (19) for the 2024 African outbreak, characterized by elevated community transmission persisting across multiple months. Importantly, this plateau in positivity, rather than the epidemic peak-and-decline dynamics typical of non-endemic outbreaks, suggests that WBS metrics in this setting are capturing endemic transmission maintained by an established reservoir in the population, not a transient outbreak signal. This has direct implications for interpreting the correlation structure: where clinical positivity is persistently high and exhibits weak autocorrelation ($r_1 = -0.34$, indicating a largely non-autocorrelated series), short-term variations in wastewater concentration are likely to reflect real fluctuations in community viral burden rather than noise, lending additional credibility to the Bugolobi FSTP lead-lag result.

Prior to this study, no peer-reviewed MPXV WBS data from SSA had been published (39). The entirety of the published WBS literature for Mpox, spanning studies from the Netherlands (16), France (17), Spain (31), the United States (10,18,30,40), Canada (15), Switzerland (37), Chile (41), and China (42) originates from settings with substantially higher wastewater infrastructure coverage, continuous-flow monitoring, and clinical surveillance density than Kampala. The present study, therefore, addresses a direct evidentiary gap at the geographic epicenter during the global PHEIC and demonstrates that MPXV WBS is not exclusively a tool for high-income settings.

The detection rates and concentration ranges observed in this study are broadly comparable to those reported for MPXV WBS in higher-resource settings. Nakivubo WWTP Inlet 1 achieved a 93.8% detection rate, among the highest reported for any MPXV WBS study to date, consistent with its likely large contributing catchment population and the sustained community transmission reflected in clinical data. The high ND frequencies at Naalya WSP (46.9%) and Nakivubo WWTP Inlet 2 (59.4%) are more consistent with prior reports from lower-prevalence or lower-catchment settings (30,37,43) and plausibly reflect genuine spatial heterogeneity in transmission intensity across Kampala sub-catchments, in addition to the analytical sensitivity constraints inherent in dPCR-based approaches for large-volume DNA targets such as the MPXV dsDNA genome.

The proof-of-concept for WBS as a leading epidemiological indicator was first rigorously established in the Netherlands for SARS-CoV-2 by Medema et al. (32) and subsequently replicated across dozens of national surveillance programmes (33,44–47). A conceptually analogous precedent exists in the use of environmental poliovirus surveillance, which has for decades provided early detection of poliovirus circulation in populations with high immunization coverage and asymptomatic shedding individuals (48,49), a paradigm that is directly relevant to MPXV, where a substantial proportion of clade Ib infections appear to present with mild or atypical symptoms, reducing the sensitivity of passive clinical surveillance (25,50). The present study extends this body of evidence to a dsDNA virus in an LMIC context, demonstrating that the core epidemiological utility of WBS, its capacity to aggregate population-level shedding signal without depending on individual healthcare contact, is not abrogated by differences in infrastructure, pathogen biology, or clinical surveillance capacity.

The significant concentration difference between Nakivubo WWTP Inlets 1 and 2 (Wilcoxon $W = 8.00$, $p_{\text{Holm}} = 0.037$) warrants comment. The two inlets drain spatially proximate areas within the same treatment works, yet Inlet 1 consistently exhibited higher MPXV concentrations. This divergence may reflect differences in the demographic composition or sexual network density of contributing catchments, or in the hydraulic characteristics of the inlet configurations themselves, specifically, differential dilution by stormwater inflow or infiltration. Analogous within-city heterogeneity has been documented in SARS-CoV-2 WBS studies employing sub-catchment sampling designs (51,52) and is increasingly recognized as a feature rather than a confound of multi-site WBS, providing granular spatial resolution that aggregate city-level surveillance cannot supply.

Several limitations temper the conclusions that can be drawn from this study and identify clear priorities for future investigation. First, the absence of continuous wastewater flow data and catchment population estimates precluded hydrological normalization of MPXV concentrations, a recognized analytical limitation in LMIC WBS contexts (29,36,53) where mass-balance normalization to population-weighted flow is standard practice in high-income settings (54,55). Although PMMoV, measured as a process control in this study, offers an

alternative normalization surrogate that corrects for within-facility variation in fecal loading (54,56), its observed temporal stability across facilities over the study period argued against its use as a concentration denominator in the primary analysis. Future deployments should integrate automatic sampler flow-pacing, continuous flow logging, and crAssphage quantification (57) as complementary normalization strategies to improve the quantitative comparability of concentration estimates across facilities and time.

Second, the limited detection weeks at Nakivubo WWTP Inlet 2 ($n = 13$) and Naalya WSP ($n = 17$) substantially constrained inferential power. Minimum detectable Spearman correlations exceeded 0.60 at three of four facilities. Null lag-0 results at those sites are therefore inconclusive rather than negative. This power limitation is an inherent challenge in ecological WBS time series with short observation windows, a problem identified across the SARS-CoV-2 WBS literature (34,58) and addressed here through pre-specified reporting of MDEs and autocorrelation-adjusted effective sample sizes. Extending the surveillance window, increasing the sampling frequency to twice weekly, or aggregating the signal across facilities would substantially improve statistical power in future iterations.

Third, substantial lag-1 autocorrelation in the wastewater series at Nakivubo WWTP Inlet 1 ($r_l = 0.657$) and Inlet 2 ($r_l = 0.765$) reduced effective sample sizes to as few as six observations after Quenouille-Bartlett correction, rendering p-values from these facilities unreliable for inferential purposes. This likely reflects the persistence of MPXV DNA over multiple weeks, consistent with reported shedding durations of up to four weeks (26,27), combined with the smoothing effect of the hydraulic residence time within the treatment works. Future analyses employing autoregressive integrated moving-average (ARIMA) models or dynamic regression approaches would explicitly account for this autocorrelation structure rather than treating it as a post hoc correction.

Fourth, the ecological study design, which correlates population-level wastewater signals with aggregate clinical positivity rather than individual shedding profiles, precludes causal inference and is susceptible to ecological fallacy. The clinical positivity metric is also confounded by testing rates: changes in healthcare-seeking behavior or diagnostic capacity can alter positivity independently of underlying transmission intensity. Triangulation with incidence-based metrics, hospitalization counts, or genomic surveillance data would strengthen causal interpretation in future work.

Finally, the Naalya WWSP lag -4 result, in which clinical positivity appears to precede the wastewater signal by four weeks ($r_s = 0.649$, $R^2 = 0.535$), is the most difficult to interpret mechanistically. A lag of this magnitude could reflect several non-exclusive processes: reporting lags, in which cases detected in a given clinical week reflect infections occurring weeks earlier; spatial mismatch between the Naalya catchment and the population contributing to reported clinical cases; or seasonal variation in wastewater flow volume that episodically dilutes or concentrates the signal. Pending the collection of flow data and geospatial case mapping aligned with treatment catchments, this result should be treated as hypothesis-generating rather than confirmatory.

Notwithstanding these limitations, the results provide a compelling empirical basis for investing in sustained, multi-site MPXV WBS in Kampala and by extension in other major urban centres across the East African clade Ib transmission network. Three features of the current epidemiological context make this investment particularly urgent. First, the former PHEIC designation (19,21) creates an international political and financial architecture for the

WBS scale-up that did not exist during the 2022 clade IIb outbreak. Second, clinical surveillance capacity in Uganda and neighboring countries remains insufficient to fully characterize transmission dynamics, creating precisely the ascertainment gap that WBS is most suited to address (28,29,36). Third, the concentration dynamics observed here, spanning more than three orders of magnitude (2.74 to 6.85 log¹⁰ GC/100 mL) across facilities and time, indicate that the quantitative wastewater signal is sensitive enough to track community-level fluctuations in viral burden, not merely to confirm presence or absence.

The success of environmental poliovirus surveillance in low-income African settings (48,49) offers an instructive parallel programme that demonstrated that regular environmental sampling at strategic drainage points, even without flow normalization or extensive laboratory infrastructure, can provide reliable, actionable public health intelligence. A similarly pragmatic, strategically-sited MPXV WBS network prioritizing high-flow, high-catchment inlet points such as Nakivubo WTP Inlet 1, and building on the analytical protocols developed in this study, represents a tractable and cost-effective complement to clinical surveillance that could provide 2–3 weeks of advance warning to public health responders.

More broadly, this study contributes to the growing evidence base supporting WBS as a scalable, pathogen-agnostic early-warning system for emerging and re-emerging infections in urban populations (46,47,53,58). The demonstration that MPXV DNA can be reliably quantified from untreated citywide and municipal influent using standard RT-qPCR infrastructure, without specialized concentration equipment or cold-chain-sensitive reagents, substantially lowers the barrier to deployment in LMIC settings. Combined with the methodological framework developed here for handling ND data, autocorrelation-adjusted inference, and facility-specific lead-lag estimation, these results provide a replicable template for MPXV WBS across the endemic zone of East and Central Africa.

In conclusion, this study demonstrates that WBS for MPXV is both technically feasible and epidemiologically informative in Kampala, Uganda, providing the first such evidence from sub-Saharan Africa. The identification of a biologically plausible 3-week early-warning signal at Bugolobi FSTP, sustained detection over 32 weeks of observation, and significant within-city heterogeneity in viral concentration dynamics together support expanding MPXV WBS as a population-level surveillance tool in the East African clade Ib epicenter. The limitations identified, most critically the absence of flow normalization data, high non-detection frequencies at lower-catchment facilities, and the short observation window, define a clear agenda for the next phase of surveillance investment. We recommend integrating flow monitoring at all sampled facilities, extending the surveillance window over the coming years, and applying the validated analytical framework described here to neighboring cities within the clade Ib transmission network. Doing so would transform wastewater from a confirmatory surveillance adjunct into a genuine early-warning system capable of guiding proactive outbreak response in the region most severely affected during the Mpox PHEIC.

METHODS

Study design and setting

We conducted a longitudinal ecological analysis using paired weekly wastewater and clinical Mpox surveillance data collected between epidemiological weeks 6–37 (February–September) in Kampala, Uganda. Wastewater samples were obtained from 4 selected WWTPs in Kampala: Naalya Wastewater Stabilization Ponds (Latitude: 0.3803140 °; Longitude: 32.6352669 °),

461 Bugolobi Fecal Sludge Treatment Plant (Latitude: 0.3194585 °; Longitude: 32.6065260 °), and
 462 the inlets of Nakivubo New WWTP (Latitude: 0.3195990 °; Longitude: 32.6078289 °) at points
 463 1 and 2 as shown in **Error! Reference source not found.** (Figure 5). These facilities serve
 464 diverse catchments, including centralized wastewater treatment, decentralized sludge
 465 treatment, and stabilization pond systems, thereby covering approximately 32% of Kampala's
 466 population (1,797,722), with the majority lacking access to sewerage systems.

467 *Table 2: Selected WWBS Pilot Sampling Sites and Population/ areas served. (Source: National*
 468 *Water and Sewerage Cooperation, Kampala, Uganda.)*

S/N	Selected WWBS Pilot Sampling Sites.	Estimated population served	Suburbs in the Drainage Reticulation Area
1	Bugolobi FSTP	220,000	Kololo, Mulago, Cesspool emptiers
2	Naalya WSP	8,000	Naalya Estates, Sunset Apartments, Kiwatule
3	Nakivubo WWTP Inlet 1	266,667	Naguru, Ntinda, Nakawa, Bugolobi, Kyambogo, Kiwanataka, Banda, Kasokoso, Butabika, Makerere, Mutungo, Central Kampala
4	Nakivubo WWTP Inlet 2	88,889	Industrial area, part of Bugolobi and Namuwongo



Figure 5: Map of Uganda showing the sampling sites.

Sample and data collection

Modified Moore swabs were prepared locally following the standard Moore swab preparation protocol (59) and deployed weekly at each site, between 9:00 a.m. and 11:00 a.m. Swabs were fully submerged in influent wastewater for 24 hours to maximize contact before retrieval. After removal, swabs were packaged in Ziploc bags, sealed twice, and externally decontaminated with 70% ethanol. These were then placed in a bio-bottle and transported on ice to the Central Emergency Response and Surveillance Laboratory (CERSL) within Uganda's Department of National Health Laboratory and Diagnostic Services (NHLDS), under the Ministry of Health, using a sample transport carrier. In total, 128 Moore swabs were deployed and collected across the four WWTPs over the eight-month period. Corresponding weekly Mpox clinical case data for healthcare facilities in Kampala were obtained from routine surveillance records maintained by the Ministry of Health's Division of Health Information and summarized at the city level.

Sample processing, Viral DNA concentration, and extraction

Sample processing and testing were performed at the CERSL. Upon receipt in the laboratory, all samples were refrigerated at 4 °C and processed within 2 hours. All liquid from the Moore swab was collected in a sterile flask, yielding approximately 200 mL of wastewater, which was then processed during the capture-and-concentration step. A 35 mL aliquot of wastewater was transferred into a sterile 50 mL Falcon tube. Then, 100 µL of Nanotrap® Enhancement Reagent 2 (ER2) (Ceres Nanosciences, Manassas, VA, USA) was added and mixed by inversion. Subsequently, 525 µL of Nanotrap® Microbiome A Particles was added, and the tube was inverted twice to mix. The sample was incubated at room temperature for 30 minutes, with inversion every 5 minutes to maintain suspension. The tube was then placed on a DynaMag-50 magnetic rack for 10 minutes to pellet the Nanotrap particles, and the supernatant was

carefully discarded without disturbing the pellet. The pellet was resuspended in 1 mL of Nanotrap Buffer 2 by gently pipetting along the tube walls until fully resuspended. The suspension was transferred into a 2 mL microcentrifuge tube and placed on a DynaMag™-2 magnetic rack for 2 minutes. Finally, the supernatant was carefully removed using a pipette, leaving the Nanotrap particle pellet for downstream processing. The Nanotrap particle pellets were lysed and purified using the QIAamp Viral DNA Mini kit (Qiagen, Hilden, Germany), which uses a conventional lysis method that rapidly releases viral DNA while simultaneously inactivating nucleases in the sample matrix. Nucleic acids were eluted to a final volume of 200 µL.

Quantitative Polymerase Chain Reaction (qPCR)

MPXV quantification was performed using the BioPerfectus Monkeypox Virus Real-Time PCR kit (BioPerfectus Technologies, Jiangsu Province, China). The specific primer and probe sequences are proprietary and not disclosed by the manufacturer; however, the primer/probe design targets the conserved F3L gene of MPXV (NCBI Gene ID: 928,998) and is conceptually similar to published MPXV F3L assays (60). The reaction master mix was prepared following the manufacturer's instructions. Briefly, 20 µL of BioPerfectus Monkeypox Virus Real-Time PCR reaction master mix, which includes primers and probes at different concentrations, was added to each well of the plate. Subsequently, 5 µL of extracted nucleic acid, nuclease-free water for the no-template control (NTC), and positive and negative controls, were added to the assay wells containing the master mix. In the Plate Setup window of the Bio-Rad CFX96 Touch system, FAM and VIC served as reporter dyes for the viral targets of the primers and probes, specifically MPXV and Internal Control (IC). The thermal cycling conditions consisted of 1 cycle at 95 °C for 5 minutes, followed by 45 cycles at 95 °C for 10 seconds and 58 °C for 30 seconds. qPCR results were analyzed using the Bio-Rad CFX Manager Dx software. Genome copy quantification per reaction (GC/reaction) for the F3L target gene was determined using a linear regression equation derived from a standard curve generated from the mean of three replicates of six tenfold serial dilutions (10^5 to 10^0) of a known Mpox DNA viral standard. The reported F3L curve had an R^2 value of 0.9996 (slope = -3.32, y-intercept = 40.15, amplification efficiency = 100%).

Quality control

Primers and probes specific to Pepper Mild Mottle Virus (PMMoV) (Promega, USA) were used as a fecal indicator and for analytical process control. The GoTaq Enviro PMMoV Quant Kit and Quasar 670 reaction master mix were prepared according to the manufacturer's instructions. A total of 20 µL of RT-qPCR reaction mixture targeting PMMoV was prepared for each well. The amplification mix consisted of 10 µL of GoTaq® Enviro Master Mix (2X), 0.4 µL of GoScript™ Enzyme Mix, 1 µL of PMMoV Primer/Probe Mix (Quasar® 670, 20X), and 3.6 µL of nuclease-free water. A total of 5 µL of the extracted nucleic acid was added to the master mix, bringing the final volume to 20 µL for the RT-qPCR reaction. In the Plate Setup window of the CFX96™ system, Quasar® 670 was used as the reporter dye for the PMMoV viral target. Thermal cycling was performed at 45 °C for 15 minutes, followed by 95 °C for 2 minutes, then 40 cycles of 95 °C for 15 seconds and 62 °C for 60 seconds.

Data preprocessing and non-detect treatment

Wastewater MPXV data from 4 WWTPs (February to September 2025) were cleaned and harmonized with corresponding weekly Mpox case numbers for clinical positivity rates (proportion of tested individuals with a positive MPXV result within the clinical surveillance

network) in the Kampala Metropolitan area using Microsoft Excel. Genomic copies per 100 mL were derived from genomic copies per reaction without PMMoV normalization, as PMMoV served solely as a process control. This decision reflected the observed temporal stability of PMMoV signals and the absence of wastewater flow and catchment population data, which precluded robust hydrological normalization. For inferential analyses conducted on the $\log_{10}$ scale, including correlation, regression, and lead-lag analyses, weeks with NDs were excluded using listwise deletion. For descriptive tabulation, ND samples were assigned a GC/100 mL value of 0; they were not included in Spearman correlations, regression models, or lead-lag analyses.

Statistical Analysis

Descriptive statistics were computed for weekly $\log_{10}$ -transformed MPXV concentrations (detected weeks only) and for weekly clinical Mpox positivity across the full study period. Distributional normality of $\log_{10}$ MPXV concentrations was assessed at each facility using the Shapiro-Wilk test. These results informed the selection of non-parametric methods as primary estimators throughout the analysis. Differences in weekly MPXV concentrations between facilities were assessed using pairwise Wilcoxon signed-rank tests applied to all available paired weeks for each facility dyad, with Holm correction to control the family-wise error rate.

The effective sample size for Spearman correlation at each facility was calculated as the number of weeks with a detectable wastewater signal after ND exclusion. Before interpreting correlation results, minimum detectable Spearman correlations (MDE) at 80% power (two-sided $\alpha = 0.05$) were calculated for each facility's effective n . At facilities with MDE above 0.60, indicating that only strong associations are detectable, findings were interpreted accordingly, and null results were not interpreted as the absence of an effect. To address inflation of nominal significance due to autocorrelation, lag-1 autocorrelation was assessed in wastewater and clinical time series; effective sample sizes were adjusted with the Quenouille-Bartlett correction, and unadjusted Spearman p -values and autocorrelation-adjusted estimates were reported. Associations between weekly $\log_{10}$ -transformed wastewater MPXV concentrations and weekly clinical Mpox positivity were assessed for each facility using Spearman's rank correlation at lag 0 (same week). Approximate 95% confidence intervals for all Spearman correlations were computed using Fisher's Z transformation.

Exploratory lead-lag analyses involved shifting clinical positivity data by ± 4 weeks relative to wastewater data within each facility. Positive lags mean wastewater leads; negative lags mean clinical leads. This window accounts for viral shedding, wastewater transport, healthcare-seeking, and reporting delays. The false discovery rate was controlled with the Benjamini-Hochberg procedure. Interpretation focused on effect size, confidence intervals, directional consistency, and biological plausibility, not just statistical significance, aligning with established WBS practices. The best lag per facility was the one with the highest absolute Spearman r_s within ± 4 weeks, with ties broken by considering $p < 0.20$ for sensitivity, given limited power in early-warning surveillance.

Effect sizes were estimated using Theil-Sen robust regression as the primary estimator, with ordinary least squares (OLS) regression reported for comparison. Weekly clinical Mpox positivity was modeled as a function of $\log_{10}$ wastewater MPXV concentration at lag 0 and at facility-specific empirically identified lags. Robust 95% confidence intervals were derived via bootstrap resampling (1,000 iterations). Population-averaged associations across facilities were estimated using generalized estimating equations (GEE) with an exchangeable working correlation structure, treating facility as the clustering variable. Models were fitted with both

585 uniform lag-0 and facility-specific lag specifications, the latter of which was treated as
586 exploratory.

587 **Statistical Analysis**

588 All analyses were conducted using Python 3.13.7 in Jupyter Notebook (Anaconda Navigator).
589 Data manipulation used pandas and NumPy; statistical analyses used SciPy and statsmodels;
590 and visualizations were generated using Matplotlib and seaborn. Two-sided p-values <0.05
591 were considered statistically significant for primary analyses, with false discovery rate-
592 adjusted thresholds applied to lead-lag analyses. Given the exploratory nature of lead-lag
593 analyses, findings were interpreted with emphasis on effect size, uncertainty, and directional
594 consistency rather than statistical significance alone. (32,33)

595 **Ethical statement**

596 Permission to use the reported Mpox clinical case data and to access the wastewater sanitation
597 systems in the Kampala Metropolitan area was obtained from the Ministry of Health's Division
598 of Health Information and the National Water and Sewage Corporation, respectively. The
599 Uganda National Health Laboratory Services Research Ethics Committee (UNHLS REC)
600 reviewed and approved the use of aggregated surveillance data for this manuscript, along with
601 a waiver of informed consent, since the data contained no personally identifiable information
602 and posed no risk to the confidentiality or dignity of the reported cases. All procedures were
603 carried out in accordance with the Declaration of Helsinki, relevant institutional policies, and
604 national regulations governing the secondary use of de-identified health data.

605 **Availability of data and material**

606 The data generated (Source data) or analyzed during this study are included in the manuscript
607 and supplementary files.

608 **Code availability**

609 All code used to generate the results reported in this study is publicly available at Zenodo
610 (<https://doi.org/10.5281/zenodo.18854960>). The repository contains a fully annotated Jupyter
611 Notebook that reproduces all statistical analyses using open-source Python libraries.

612 **Abbreviations**

613 ANOVA: Analysis of Variance

614 CDC: Centers for Disease Control and Prevention

615 CERSL: Central Emergency Response and Surveillance Laboratory

616 CFR: Case Fatality Rate

617 CI: Confidence Interval

618 Ct: Cycle threshold

619 DNA: Deoxyribonucleic Acid

620 ER2: Enhancement Reagent 2

621 FAM: 6-Carboxyfluorescein (Fluorescent Dye)

622 FSTP: Faecal Sludge Treatment Plant

623 GC: Genome Copies

624 IC: Internal Control

625 LOD: Limit of Detection

626 MPXV: Monkeypox/ Mpox Virus

627 NHLDS: Department of National Health Laboratory and Diagnostic Services

628 NTC: No-Template Control

629 PCR: Polymerase Chain Reaction

630 PMMoV: Pepper Mild Mottle Virus

631 qPCR: Quantitative Polymerase Chain Reaction

632 R²: Coefficient of Determination

633 VIC: Fluorescent dye

634 WBS: Wastewater-Based Surveillance

635 WHO: World Health Organization

636 WSP: Wastewater Stabilization Pond

637 WWTP: Wastewater Treatment Plant

638 **Declaration**

639 **Clinical trial**

640 Not applicable

641 **Consent for publication**

642 Not applicable

643 **Competing interests**

644 All authors declared no competing interests.

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Authors contributions

Protocol development and conceptualization: AN and IS. Methodology: AN, VN, and JK. Sample collection and analysis: GMB, IM, and JM. Data analysis: JK and SJM. Data curation and validation: AN and JK. Original manuscript writing: AN, GMB, IM, and VN. Technical supervision: VN. Funding: FC. Manuscript editing and final review: All authors contributed equally.

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Supplementary file: Source Data and Data Analysis outputs

Supplementary Files

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