Mpox Seroprevalence among Sex Workers and Men who have Sex with Men Preceding Sustained Urban Outbreak in the Democratic Republic of the Congo

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Abstract:	Mpox has rapidly evolved into a global public health concern following rapid geographic expansion and altered clinical and epidemiological characteristics. The 2022 global outbreak of monkeypox virus (MPXV) marked the first widespread expansion to non-endemic regions, particularly among urban communities of men who have sex with men (MSM). In the Democratic Republic of the Congo (DRC), where Clade I MPXV remains endemic, data on urban exposure are limited. We evaluated serologic evidence of MPXV exposure among 1,823 participants from two major DRC cities, Kinshasa and Goma, between April and June 2024. Of these participants, 523 (28.7%) self-identified as MSM, 498 (27.3%) as professional sex workers (SW), 107 (5.9%) as MSM and SW, and 695 (38.1%) as other at-risk individuals based on occupation or recruitment location. Serum samples were evaluated using a four-MPXV antigen multiplex assay. Overall, 2.9% of participants were seroreactive. By subpopulation, 4.8%, 2.1%, and 1.7% of at-risk, MSM, and SW participants were seroreactive, respectively ($p = 0.0086$). Classification tree modelling identified age as the strongest predictor of reactivity (when compared with selected zoonotic and sexual risk factors), with higher MPXV-seroreactivity in older participants. These data reveal baseline mpox seroreactivity prior to sustained human-to-human transmission in urban settings. Understanding exposure dynamics is critical for anticipating re-emergence of mpox in global cities where interpersonal networks may sustain transmission even in the absence of zoonotic spillover.

Mpox Seroprevalence among Sex Workers and Men who have Sex with Men Preceding Sustained Urban Outbreak in the Democratic Republic of the Congo

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ABSTRACT

Mpox has rapidly evolved into a global public health concern following rapid geographic expansion and altered clinical and epidemiological characteristics. The 2022 global outbreak of monkeypox virus (MPXV) marked the first widespread expansion to non-endemic regions, particularly among urban communities of men who have sex with men (MSM). In the Democratic Republic of the Congo (DRC), where Clade I MPXV remains endemic, data on urban exposure are limited. We evaluated serologic evidence of MPXV exposure among 1,823 participants from two major DRC cities, Kinshasa and Goma, between April and June 2024. Of these participants, 523 (28.7%) self-identified as MSM, 498 (27.3%) as professional sex workers (SW), 107 (5.9%) as MSM and SW, and 695 (38.1%) as other at-risk individuals based on occupation or recruitment location. Serum samples were evaluated using a four-MPXV antigen multiplex assay. Overall, 2.9% of participants were seroreactive. By subpopulation, 4.8%, 2.1%, and 1.7% of at-risk, MSM, and SW participants were seroreactive, respectively (p = 0.0086). Classification tree modelling identified age as the strongest predictor of reactivity (when compared with selected zoonotic and sexual risk factors), with higher MPXV-seroreactivity in older participants. These data reveal baseline mpox seroreactivity prior to sustained human-to-human transmission in urban settings. Understanding exposure dynamics is critical for anticipating re-emergence of mpox in global cities where interpersonal networks may sustain transmission even in the absence of zoonotic spillover.

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Data Availability Data will be made available upon reasonable request to the corresponding author.

Conflict of Interest None to declare.

Ethical Statement This study was approved by the Institutional Review Boards of the University of California, Los Angeles (IRB#23–000676), University of Manitoba (HS25837) and the Kinshasa School of Public Health, University of Kinshasa, DRC (ESP/CE/161/2024).

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INTRODUCTION

Mpox (previously, monkeypox), is a zoonotic viral disease endemic in tropical forested regions of Central and West Africa, with the Democratic Republic of the Congo (DRC) among the most affected countries. Since reporting the first human case in 1970 [1], the DRC has experienced substantial increases in mpox incidence, with over 10,000 laboratory-confirmed cases reported in 2024 alone [2-5]. Historically, human mpox incidence has been driven by zoonotic events with self-limiting transmission clusters in ~~rural~~ remote regions, where rodents were believed to play a key role as a potential reservoir [6].

Genomically, monkeypox virus (MPXV) has been divided as two separate clades: Clade I, endemic in regions of the Congo River Basin, and Clade II, endemic in Western African regions [7]. While studies have shown virulence differences between the two clades, more recent observations from mpox outbreaks have been less certain. Both virus clades have demonstrated transmission through similar zoonotic events, and increasing persistence in human populations, with seemingly context-specific divergence [8]. Though mpox outbreaks had previously been largely confined to endemic regions of Africa, the 2022 global outbreak marked a major epidemiological shift, with rapid international spread of Clade IIb among non-endemic regions, concentrated among men who have sex with men (MSM) [9,10]. Unlike earlier zoonosis-driven outbreaks, transmission during this outbreak was sustained through human-to-human spread within sexual networks and the absence of zoonotic exposures [11]. Furthermore, in 2024, a novel sub-lineage of Clade I was identified—Clade Ib—with sustained human-to-human transmission clusters first detected in Kamituga, South Kivu Province, DRC [4]. Subsequent work delineating Clade I MPXV diversity has shown a predominance of zoonotic transmission in DRC related to Clade Ia, historically comprising Clade I, with specific sexual networks and high population density contributing to the continued propagation of mpox (Clade Ib) in urban centers [12].

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Today, outbreaks of mpox in urban settings continue to be sporadically reported both in endemic and non-endemic settings; for instance, as of October 2025, Clade I MPXV was confirmed in Los Angeles County, California in patients with no reported travel to endemic regions, yet later linked to an importation event with evidence of local transmission [13,14]. While continued mpox emergence and sustained transmission was historically unprecedented in non-endemic settings, these recurrent events highlight the need to examine transmission dynamics in urban centers in mpox-endemic countries to clarify whether similar network-driven mechanisms occur or whether distinct social, ecological, or virological factors sustain mpox transmission in these regions.

Recent studies have highlighted the increasing role of sexual transmission in mpox outbreaks in the DRC. A notable cluster of Clade Ia infections in March 2023 associated with sexual or intimate contact first suggested that this new mode of transmission was not unique to the Clade IIb variant [15]. In Kamituga, South Kivu Province, an urban outbreak in late 2023 identified 29% of PCR-confirmed cases as female sex workers, with 61% of suspected cases as sexually active and reporting multiple partners, all supporting the hypothesized role of sexual-contact driven transmission [4]. The prevalence of APOBEC3-mediated mutations in Clade Ib MPXV further suggested ongoing human-to-human transmission [16]. Concurrently, mounting evidence suggests that human-to-human chains are now sustaining outbreaks even in non-endemic regions. Molecular and serological screening in Central Africa and Europe have uncovered both symptomatic and asymptomatic MPXV infections in sexual health clinics, implying that clinical surveillance may vastly underestimate true transmission—particularly within dense sexual networks [17,18].

Widespread mpox surveillance in the DRC is limited to those symptomatic cases identified at local health centers and reported passively through structures such as the Integrated Disease and Response (IDSR) System and to the National Program for the Control of Mpox and Viral

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Hemorrhagic Fevers (French acronym, PNLMPX-VHF) [19]. Yet, these systems are resource-limited and support few research-driven, case-specific outbreak investigations. Furthermore, contact-tracing and confirmatory laboratory testing are even more restricted due to historical underfunding and logistical challenges associated with biological specimen collection and appropriate transport [20]. While during the declared Public Health Emergency of International Concern, additional resources were mobilized to conduct active surveillance based at the National Institute for Biomedical Research (French acronym, INRB), this activity was specifically initiated in response to World Health Organization declarations. Given these constraints, seroprevalence studies are valuable approaches to understand the extent of mpox exposure and population immunity and to identify potential risk factors [21,22].

Considering the epidemiologic shifts of mpox transmission and those sub-populations considered most at-risk globally, as well as the presumed underreporting of mpox in DRC, this study sought to determine prevalence of anti-MPXV antibodies among suspected high-risk populations in urban centers. Specifically, this study assessed seroprevalence among those populations most affected by the 2022 global outbreak, those vulnerable due to dense sexual networks and limited access to health care due to social stigma: MSM and professional sex workers (SW). Furthermore, sampling also included those populations at increased risk due to zoonotic exposure through their occupation or other reported lifestyle habits. Outside of DRC, previous serosurveillance studies in Kenya have indicated high mpox seropositivity rates in these groups in the absence of reported infections, indicating significant underreporting of urban mpox cases [23].

METHODS

Population Recruitment and Sample Collection

This cross-sectional study sought to determine the seroprevalence of mpox among key populations in two geographically distinct urban centers of DRC: Kinshasa (Kinshasa Province) and Goma (North Kivu Province). These locations were selected due to their high population density and mobility, factors that elevate the risk of MPXV transmission. Between April and June 2024, participants were recruited from three suspected at-risk sub-populations: self-identified MSM, self-identified SW, and other at-risk members of the general population based on their location and occupation. At the time of enrollment, mpox-specific vaccines were not available for administration.

In order to enroll these often socially subtle, at-risk groups, recruitment strategies relied on a combination of time-location (TLS) and snowball sampling. Peer educators (PEs), who were members of the MSM and SW communities, played a key role in the recruitment process [24]. In collaboration with the National Program for the Fight against HIV/AIDS and Sexually Transmitted Infections (French acronym, PNLS) and PEs, key locations frequented by these populations, such as health facilities, bars, nightclubs, markets and city entrances, were identified for sampling.

Eligible participants were recruited if they were ≥18 years old and self-identified as either a MSM or SW. Additionally, at PE-directed sampling sites, non-MSM and non-SW were enrolled as a convenience sample of an otherwise at-risk population (ARP); the ARP also included those participants recruited at farms and abattoirs (suspected occupational zoonotic exposure). All participants consented to both survey participation and blood sampling [24]. This study was approved by the Institutional Review Boards of the University of California, Los Angeles (IRB#23–000676), University of Manitoba (HS25837) and the Kinshasa School of Public Health, University of Kinshasa, DRC (ESP/CE/161/2024).

Data collection consisted of two main components: an interviewer-led semi-structured questionnaire, and serum collection at enrollment. The survey instrument, hosted on a secure survey platform server (*SurveyCTO* (Version 2.0), Dobility, Inc), included questions on

demographic characteristics (e.g. age, education, marital status), sexual and clinical history, knowledge of mpox transmission, and zoonotic and sexual risk factors. Additionally, trained phlebotomists collected a venous blood sample that was aliquoted in the field into serum, and plasma and buffy coat samples.

Serologic Testing

Serum samples were evaluated for reactivity using a four-plex immunoassay of three synthetic peptides derived from the right terminal region B of MPXV, B21R.179/180, B21R.185/186, and B22R.64/65 [25], as well as the highly-conserved *Poxviridae* peptide, H3L [26,27]. In brief, each peptide was first conjugated to bovine serum albumin before covalent attachment to magnetic beads with distinct fluorescent signatures (Luminex Corp, Austin, TX, USA). A master-mix bead solution containing Phosphate Buffered Saline (PBS) with 0.75 mol/NaCl, 5% heat-inactivated fetal bovine serum (Gibco-Invitrogen, France), 1% bovine serum albumin, and 0.2% Tween-20 (Sigma-Aldrich, France) was added to each well of 96-well flat-bottom chimney plates (Greiner Bio One, Germany). Diluted serum samples (1:200) were subsequently added to individual wells in duplicate; following washing steps, a secondary biotin-labeled anti-anti-human IgG antibody (BD-Pharmingen, France) was added. Finally, streptavidin-R-phycoerythrin (Fisher Scientific/Life Technologies, France) was introduced; with antigen-antibody binding quantified as median fluorescence intensity (MFI) per 100 beads using a Magpix instrument (Bio-Rad, France). All sample testing was completed on-site in Kinshasa, DRC.

As described by Kinganda-Lusamaki et al., the mean MFI plus three standard deviations of an 90-person mpox-negative panel was calculated as the respective seroreactivity threshold for each of the MPXV-specific peptides, B21R.179/180, B21R.185/186, and B22R.64/65 [18]. Given the unknown orthopoxvirus vaccination status of the 90-person panel, the cut-off value for anti-H3L reactivity was calculated using the likelihood ratio from a Receiver Operating Characteristic (ROC) curve (Supporting Information: Figure S1 of the original publication[18]).

Seroreactivity results were binarized, calculated by dividing the MFI value of each sample by the corresponding cut-off for each peptide; an index >1 was considered seroreactive. As positivity to any individual peptide of the four-plex was deemed sufficient for a seroreactive result [25], the maximum index value was considered for each sample.

Statistical Analysis

All data analysis was completed with SAS version 9.4 (SAS Institute, Cary, NC) and R version 4.3.3 (R Foundation for Statistical Computing, Vienna, Austria). Descriptive statistics were generated for participant demographics and seroreactivity rates. Unadjusted measures of association between categorical variables were assessed using chi-square tests, while continuous variable associations were evaluated with two-tailed Student's t-tests. Statistical significance was determined using p-values ($p < 0.05$), and 95% confidence intervals (CI) were reported to assess the precision of the estimates.

Given the spread of zoonotic and sexual risk factors and unadjusted associations with detected seroreactivity, a five-point composite score was generated for both zoonotic and sexual risk **(Figure 1)**. Variables included in these scores were selected based on completeness as well as *a priori* understandings of mpox transmission through either zoonotic or human-to-human routes. Zoonotic risk scores were calculated based on the binary responses to the following statements, with each "yes" response equating to one point on the risk scale: 1) Have you ever visited a forested/wooded area?; 2) Have you ever hunted wild animals?; 3) Have you ever prepared a wild animal for cooking?; 4) Have you ever consumed bushmeat?; and 5) Do you work with or interact with wild animals? Similarly, the sexual exposure risk score was calculated with the following responses: 1) Do you have a primary sexual partner? ("no" response as one point); 2) In the last three weeks have you had sexual relations with more than one person?; 3) Did you use a condom the last time you had sex? ("no" response as one point); 4) Have you ever had sex in exchange for money, drugs, food or accommodation?; and 5) a total partner count greater than

the median of the study population (as calculated by total estimated male and female partner counts).

To further understand the relative contributions of predictor variables in explaining seroreactivity results, the R packages "tree", "randomForest" and "party" [28] were used to generate classification trees and iterative regression models to identify top predictor variables and rankings of predictors contributing to explained seroreactivity (binary outcome, reactive = 1). A final model was employed that consisted of 500 trees; at each node, with 5 covariates randomly selected as splitting candidates for each tree constructed. Model fit was evaluated using an out-of-bag (OOB) error estimate and presentation of the class confusion matrix. Additionally, feature importance was calculated via a variable importance measure (mean decrease in Gini impurity, see Liaw and Wiener ~~2002~~ for details) and presented alongside a corresponding classification tree. To address class imbalance, an over-weighted model of 350 randomly selected participants including all 53 seroreactivity individuals was run; yet this only marginally improved the confusion matrix of the model. Lastly, logistic regression models to adjust for selected covariates were included to determine potential drivers of seroreactivity with Model 1 including selected demographic characteristics and the collapsed sexual risk score, and Model 2 the collapsed zoonotic risk score.

RESULTS

Overall Seroreactivity

A total of 1,823 participants were enrolled between April and June 2024— 913 (50.1%) from Kinshasa and 910 (49.9%) from Goma. Across specifically recruited sub-populations: 520 MSM (28.5%), 586 SW (32.1%), 110 MSM and SW (6.0%), and 607 ARP individuals (33.3%) were enrolled, with equivalent distributions in the two cities. Serological testing indicated an overall mpox seroprevalence of 2.9% (53/1823) among all participants, with 4.8% reactivity detected among ARP participants. In contrast, reactivity rates were lower among MSM (2.1%) and SW

(1.7%) and MSM/SW (2.7%) populations. Seroreactivity was similar across urban centers, with 3.1% detected in Kinshasa and 2.8% in Goma. By antigen, 22 participants were reactive to H3L (1.2%), 17 to B21R.185/186 (0.9%), 12 to B21R.179/180 (0.7%) and 5 to B22R.64/65 (0.3%). **(Table 1).** Of all 53 reactive individuals, only three respondents were considered reactive to more than one antigen, one reactive to H3L and B21R 180/186, and another two respondents to the both B21R antigens **(Supplementary Figure 1).** No individual was reactive to all four antigens presented.

Seroreactivity by Population Demographics and Medical History

The median age of participants was 25 years (IR: 21-32), with the majority (46.8%) aged 18–24 years and decreasing representation in older age groups **(Table 2)**. Seroreactivity was significantly associated with age (p<0.0001). Participants aged ≥45 years demonstrated markedly higher seroprevalence (11.1–19.2%) compared to younger age groups (2.2–2.9%). When binarizing age by those born before or after the cessation of smallpox vaccination campaigns[29], this difference in seroreactivity is more pronounced with those born pre-1984, reporting 7.8% reactivity (p=0.0001). While the collapsed seroreactivity variable indicates higher reactivity among those born pre-1984 (n = 12/153) , by antigen, this is almost exclusively driven by H3L reactivity (n = 11, 91.7%) **(Supplementary Figure 1).** While not statistically significant (p=0.413), heterosexual individuals had a higher overall proportion of seropositivity (3.3%). No significant difference in seroprevalence was observed based on recruitment site (e.g., bars, clubs, health facilities) (p=0.913) or among those reporting zoonotic exposures such as working at abattoirs (p=0.6914). Educational attainment was not significantly associated with seroprevalence (p=0.692), though individuals with only primary education had a slightly higher seroreactivity rate (5.3%) compared to those with secondary or university-level education (2.4–3.2%).

While not significant association was observed (p=0.247) between STI histories and seroreactivity, , trends such as higher rates of MPXV seroreactivity were observed among those

respondents who had no history of past STI diagnosis (3.5% vs 2.6%, respectively) were observed. ~~However~~Interestingly, among respondents with a self-reported history of STIs (n = 1136), those who had reported past syphilis diagnoses were more seroreactive (5/102, 4.9%) than respondents reporting other STIs. Notably, of the 25 individuals who self-identified as HIV-positive, no reactivity ~~to MPXV~~ was detected.

Seroreactivity by Zoonotic and Sexual Exposures

Beyond specific demographic characteristics or medical histories, this study sought to evaluate seroreactivity rates by general zoonotic and sexual risk factors **(Table 3)**. Seroreactivity was higher among participants reporting selected wildlife or forest contacts: 22 participants of the 458 who reported ever visiting wooded or forested areas were MPXV seroreactive (4.8%; p=0.0052); 4.3% (22/516; p=0.031) of those who prepared or cooked wild animals, as well as 3.7% of participants who reported bushmeat consumption (37/991; p=0.0232). Occupational animal contact (working at abattoirs or farms, p=0.4595) and reported personal protective equipment (PPE) use were not significantly associated with seroreactivity.

Across all participants, there was a significant association between reporting past sex with both men and women and MPXV seroreactivity: participants who reported ever having had sex with a man had lower rates of reactivity (2.3%), when compared to those who had ever had sex with a woman (4.0%). No significant associations were observed between sexual behaviors such as condom use, group sex, and ~~quid pro quo~~transactional sex, with ~~MPXV~~ seroreactivity.

The random forest classification model obtained an OOB error rate of 3.1% indicating strong predictive performance (n = 1705 complete observations). However, while the class 0 error of this model was 0.0012, the reciprocal class 1 error was 0.981, indicating poor sensitivity of the model to detect true seroreactive cases. Across both the feature importance assessment **(Figure 2)** and the generated classification tree **(Figure 3)**, the age group split of respondents younger or

older than 45 years old was identified as the predominant covariate influencing the mpox seroreactive outcome. While handling animal waste is presented as a second node of the classification tree, it is unclear as to why such exposure would have meaningfully contributed to predictive seroreactivity among a younger, urban population who participated in this study, and may simply be acting as a surrogate for more general behavioral/social conditions (i.e., it is possible that this specific question may be indicative of a more general health/hygiene discrepancy between participants or locations).

While the random forest approach incorporates potential confounders as splitting variables, this statistical method does not provide adjusted estimates for select risk factors and the outcome of binary seroreactivity. To address this, in two separate multivariate logistic regression models adjusting for sex and zoonotic risk, or population type, location, sex and age, age category was consistently identified as a significant factor associated with seroreactivity (Wald p-value = 0.0009, and 0.0021, **Table 4**). In Model 1, when adjusting for the collapsed sexual risk scores, participants aged 45-54 and 55+ had 3.92- and 7.77-times higher odds (CI 1.42-10.81; 2.37-25.52) of seroreactivity than those participants aged 18-24. Additionally, this trend of increased odds in older populations was maintained when adjusting collapsed zoonotic risk scores and other demographic confounders in Model 2: participants aged 45-54 and 55+ had 3.49- and 6.11-times higher odds than those participants aged 18-24 (CI: 1.30-9.37; 1.86-20.03).

DISCUSSION

This baseline serosurvey of high-risk urban populations provides a unique cross-sectional estimate of mpox exposure rates prior to the sustained human-to-human transmission of the virus in Kinshasa and Goma first reported in the later part of 2024. While overall seroprevalence was low (2.9%), patterns across demographic and behavioral groups offer important insights into evolving transmission dynamics.

The sample collection period of this study suggests that the detected infections likely resulted from sporadic zoonotic introductions and small, self-limiting clusters--similar to those historically documented in Central Africa--rather than the prolonged human-to-human transmission chains now observed in urban centers of DRC [30]. These initial transmission dynamics may be best described as stuttering chains with an initial spillover event seeding weak human-to-human spread [31-33]. Specifically, in Kinshasa, the first case of Clade Ib MPXV was detected in July 2024 and large-scale detection of sustained human transmission was not confirmed until mid-August 2024, well after the sampling period of this study [30,34]. Similarly, the first cases of Clade Ib MPXV were not confirmed in Goma until June 2024, at the same time that this study was conducted–[35][34]. Given the general timeline of antibody development, it is highly likely that all detected seroreactivity predates the confirmed introduction of Clade Ib MPXV in both cities. While mpox in endemic regions has been traditionally attributed to rural wildlife-to-human spillover, our findings show that such introductions may also occur in urban contexts, potentially seeding emerging transmission networks.

Seroprevalence varied across demographic groups, with older age strongly associated with seroreactivity. However, the higher prevalence among older participants could reflect cumulative lifetime exposure or residual immunity from historic smallpox vaccination campaigns. Moreover, when dividing the cohort by those born before and after 1984 (presumed vaccination), all but one of the 12 individuals identified as reactive were specifically reactive to the H3L antigen. While smallpox was officially declared as eradicated in 1980, there has been evidence of continued sporadic vaccination campaigns in the DRC up until 1984[29]. Here, we specifically chose birth year of 1984 as a more conservative cut off for potential exposure to the vaccina-based vaccine. Notably, the age split at age 45 as indicated by the regression tree aligns with a 1980 birth year cut point, potentially indicating that exposure among this group (the same 12 reactive

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individuals as identified by the 1984 cut point) is instead driven by historic orthopoxvirus vaccination.

The H3L envelope protein is a well conserved region across the *Poxviridae* family, including vaccinia virus (VACV) [27]. As this antigenic target was included as a component of the binary reactivity status, a history of VACV exposure—whether via vaccination or natural infection—may instead be driving this observed association. This potential cross-reactivity with vaccinia-derived immunity should not be understated as the potential true mechanism driving higher reactivity rates among older participants. However, as we did not specifically collect vaccinia vaccination history, respondent age serves as a proxy for this exposure where those over 45 years old are highly likely to have been vaccinated as part of smallpox eradication practices.

Yet, among a separate cohort of individuals with differential orthopoxvirus exposures, only 6/26 smallpox vaccinees were reactive to H3L (unpublished data). Previous studies have indicated that waning smallpox immunity in DRC has gradually eroded cross-protective immunity, thereby increasing the proportion of immunologically naïve individuals susceptible to mpox [5]. As the younger MSM and SW of this cohort are unlikely to have been vaccinated for smallpox, these populations are likely to suffer additional disease burden as human-to-human mpox transmission persists. The lower seroreactivity rates, and by extension, immune-naive status among MSM and SW ~~may~~ identify MSM and SW populations as high priority groups for ~~for preventative public health intervention, such as~~ mpox vaccination campaigns.

The highest seroreactivity was detected among the sampled ARP (farm workers, non-MSM and non-SW) (4.8%), a pattern consistent with traditional pathways of mpox exposure via animal-to-human contact. Although age was identified as the most predominant predictor of seroreactivity—both by the random forest and generalized linear regression model approaches—, notable associations were also observed with self-reported behaviors indicative of zoonotic risks:

specifically, bushmeat consumption and visiting forested areas. Interestingly, these activities are typically considered to be elements of rural exposure profiles; yet over 50% of respondents in Kinshasa and Goma had reported consuming wild animals at least once in their lifetime. This finding suggests that the boundaries between rural and urban exposure routes may be increasingly blurred, emphasizing the interconnected nature of zoonotic risk in the urban landscape of the DRC. Future investigations should build off these findings to better define which key factors may be driving this zoonotic risk among this ARP, as well as sampling of a true urban general population to establish a more universal urban seroreactivity threshold irrespective of sub-population by occupational or behavioral classifications. Furthermore, the sampling design of this approach further limits generalizability of these seroreactivity rates to the larger urban population; these results are limited in their applicability beyond those specific cohorts recruited for this study.

Unlike similar studies conducted in Kenya which identified strong seropositivity among SW and MSM communities in Nairobi [23], sexual risk behaviors and identification as SW or MSM were not identified as drivers of mpox seroreactivity in Kinshasa and Goma. However, these differences may be attributed to the sampling type – prospective as compared to retrospective from convenience sampling – antibody targets of the serologic assay, and other additional variables including community-specific behavioral differences. Statistical associations were only observed between reactivity and reported lifetime partner counts and frequent intimate contacts, yet these exposures did not supersede age as drivers of the reactivity outcome when assessed by the classification model. Sexual behavior patterns suggest that mixed sexual networks may facilitate early mpox spread. Heterosexual men and MSM reporting vaginal intercourse showed higher seroprevalence, indicating potential bridging between heterosexual and MSM networks. This differs from the 2022 Clade IIb global outbreak, which was largely confined to MSM populations [9].

While our iterative regression model achieved a low average error estimate, ultimately this approach had poor sensitivity in detecting seroreactive cases, most likely due to class imbalance.

Such machine-learning approaches (e.g. partitioned tree classifications, random forests) can always be improved with the inclusion of additional variables and response data; ongoing work is currently being conducted to re-enroll these same target populations to capture additional predictor variables and reassess seroreactivity.

As with any cross-sectional study design, this approach limits the ability to differentiate between recent and historic mpox exposure; lifetime exposure questions may also be subject to recall and reporting biases, leading to a possible over- or under-representation of the true exposure histories of participants. Future longitudinal sampling of these vulnerable urban populations, especially following the sustained human-to-human transmission reported in late 2024, would help to further elucidate which specific behavioral, sexual and zoonotic risks may be contributing to mpox spread in urban areas the DRC. Another limitation of this study is the binary approach to seroreactivity, while the B21R and B22R antigenic targets are MPXV-specific, the inclusion of H3L reactivity as a component of the MPXV reactivity definition may overestimate exposure. While supplementary data has demonstrated most reactivity (50/53 respondents) was to one antigen of the panel, further antigen-specific analysis should be conducted to better elucidate potential MPXV-specific exposure.

Our results suggest low-level baseline exposure to mpox among a subset of vulnerable urban populations. Of note, the four-plex MPXV serologic assay employed in this study has reported good sensitivity and high specificity to detect antibodies both early in infection, but also showed antibody reactivity decline overtime, up to two years after infection [18,36]. This facet of the assay functionality is in line with our conclusions that those detected seroreactive individuals may have higher reactivity rates with age—whether that be historic orthopoxvirus vaccination status, or more simply increased exposure frequency over time as an inhabitant of an mpox-endemic region. Ultimately, these results define a baseline exposure rate to mpox, specifically among populations suspected to be at higher risk in an urban setting.

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As Clade I MPXV continues to rapidly evolve, with increasing evidence of sustained human to human transmission of both Clades Ia and Ib [30], these results can inform context-specific behavioral and demographic vulnerabilities to guide early detection and public health intervention. While some transmission characteristics may be environmentally, behaviorally, or culturally bespoke, the MPXV transmission cycle itself is not unique to DRC, and by identifying particularly vulnerable populations in one setting, results are likely to help identify similar at-risk populations in others. Baseline serosurveys such as this are crucial for understanding the foundations of emerging epidemics. By characterizing pre-outbreak exposures and the complex interplay of zoonotic and behavioral risk factors among key populations in an endemic setting, this work provides a vital reference point for interpreting current and future urban mpox outbreaks—both in DRC, and abroad.

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Mpox Seroprevalence among Sex Workers and Men who have Sex with Men Preceding Sustained Urban Outbreak in the Democratic Republic of the Congo

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ABSTRACT

Mpox has rapidly evolved into a global public health concern following rapid geographic expansion and altered clinical and epidemiological characteristics. The 2022 global outbreak of monkeypox virus (MPXV) marked the first widespread expansion to non-endemic regions, particularly among urban communities of men who have sex with men (MSM). In the Democratic Republic of the Congo (DRC), where Clade I MPXV remains endemic, data on urban exposure are limited. We evaluated serologic evidence of MPXV exposure among 1,823 participants from two major DRC cities, Kinshasa and Goma, between April and June 2024. Of these participants, 523 (28.7%) self-identified as MSM, 498 (27.3%) as professional sex workers (SW), 107 (5.9%) as MSM and SW, and 695 (38.1%) as other at-risk individuals based on occupation or recruitment location. Serum samples were evaluated using a four-MPXV antigen multiplex assay. Overall, 2.9% of participants were seroreactive. By subpopulation, 4.8%, 2.1%, and 1.7% of at-risk, MSM, and SW participants were seroreactive, respectively ($p = 0.0086$). Classification tree modelling identified age as the strongest predictor of reactivity (when compared with selected zoonotic and sexual risk factors), with higher MPXV-seroreactivity in older participants. These data reveal baseline mpox seroreactivity prior to sustained human-to-human transmission in urban settings. Understanding exposure dynamics is critical for anticipating re-emergence of mpox in global cities where interpersonal networks may sustain transmission even in the absence of zoonotic spillover.

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Data Availability Data will be made available upon reasonable request to the corresponding author.

Conflict of Interest None to declare.

Ethical Statement This study was approved by the Institutional Review Boards of the University of California, Los Angeles (IRB#23–000676), University of Manitoba (HS25837) and the Kinshasa School of Public Health, University of Kinshasa, DRC (ESP/CE/161/2024).

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INTRODUCTION

Mpox (previously, monkeypox), is a zoonotic viral disease endemic in tropical forested regions of Central and West Africa, with the Democratic Republic of the Congo (DRC) among the most affected countries. Since reporting the first human case in 1970 [1], the DRC has experienced substantial increases in mpox incidence, with over 10,000 laboratory-confirmed cases reported in 2024 alone [2-5]. Historically, human mpox incidence has been driven by zoonotic events with self-limiting transmission clusters in remote regions, where rodents were believed to play a key role as a potential reservoir [6].

Genomically, monkeypox virus (MPXV) has been divided as two separate clades: Clade I, endemic in regions of the Congo River Basin, and Clade II, endemic in Western African regions [7]. While studies have shown virulence differences between the two clades, more recent observations from mpox outbreaks have been less certain. Both virus clades have demonstrated transmission through similar zoonotic events, and increasing persistence in human populations, with seemingly context-specific divergence [8]. Though mpox outbreaks had previously been largely confined to endemic regions of Africa, the 2022 global outbreak marked a major epidemiological shift, with rapid international spread of Clade IIb among non-endemic regions, concentrated among men who have sex with men (MSM) [9,10]. Unlike earlier zoonosis-driven outbreaks, transmission during this outbreak was sustained through human-to-human spread within sexual networks and the absence of zoonotic exposures [11]. Furthermore, in 2024, a novel sub-lineage of Clade I was identified—Clade Ib—with sustained human-to-human transmission clusters first detected in Kamituga, South Kivu Province, DRC [4]. Subsequent work delineating Clade I MPXV diversity has shown a predominance of zoonotic transmission in DRC related to Clade Ia, historically comprising Clade I, with specific sexual networks and high population density contributing to the continued propagation of mpox (Clade Ib) in urban centers [12].

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Today, outbreaks of mpox in urban settings continue to be sporadically reported both in endemic and non-endemic settings; for instance, as of October 2025, Clade I MPXV was confirmed in Los Angeles County, California in patients with no reported travel to endemic regions, yet later linked to an importation event with evidence of local transmission [13,14]. While continued mpox emergence and sustained transmission was historically unprecedented in non-endemic settings, these recurrent events highlight the need to examine transmission dynamics in urban centers in mpox-endemic countries to clarify whether similar network-driven mechanisms occur or whether distinct social, ecological, or virological factors sustain mpox transmission in these regions.

Recent studies have highlighted the increasing role of sexual transmission in mpox outbreaks in the DRC. A notable cluster of Clade Ia infections in March 2023 associated with sexual or intimate contact first suggested that this new mode of transmission was not unique to the Clade IIb variant [15]. In Kamituga, South Kivu Province, an urban outbreak in late 2023 identified 29% of PCR-confirmed cases as female sex workers, with 61% of suspected cases as sexually active and reporting multiple partners, all supporting the hypothesized role of sexual-contact driven transmission [4]. The prevalence of APOBEC3-mediated mutations in Clade Ib MPXV further suggested ongoing human-to-human transmission [16]. Concurrently, mounting evidence suggests that human-to-human chains are now sustaining outbreaks even in non-endemic regions. Molecular and serological screening in Central Africa and Europe have uncovered both symptomatic and asymptomatic MPXV infections in sexual health clinics, implying that clinical surveillance may vastly underestimate true transmission—particularly within dense sexual networks [17,18].

Widespread mpox surveillance in the DRC is limited to those symptomatic cases identified at local health centers and reported passively through structures such as the Integrated Disease and Response (IDSR) System and to the National Program for the Control of Mpox and Viral

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Hemorrhagic Fevers (French acronym, PNLMPX-VHF) [19]. Yet, these systems are resource-limited and support few research-driven, case-specific outbreak investigations. Furthermore, contact-tracing and confirmatory laboratory testing are even more restricted due to historical underfunding and logistical challenges associated with biological specimen collection and appropriate transport[20]. While during the declared Public Health Emergency of International Concern, additional resources were mobilized to conduct active surveillance based at the National Institute for Biomedical Research (French acronym, INRB), this activity was specifically initiated in response to World Health Organization declarations. Given these constraints, seroprevalence studies are valuable approaches to understand the extent of mpox exposure and population immunity and to identify potential risk factors [21,22].

Considering the epidemiologic shifts of mpox transmission and those sub-populations considered most at-risk globally, as well as the presumed underreporting of mpox in DRC, this study sought to determine prevalence of anti-MPXV antibodies among suspected high-risk populations in urban centers. Specifically, this study assessed seroprevalence among those populations most affected by the 2022 global outbreak, those vulnerable due to dense sexual networks and limited access to health care due to social stigma: MSM and professional sex workers (SW). Furthermore, sampling also included those populations at increased risk due to zoonotic exposure through their occupation or other reported lifestyle habits. Outside of DRC, previous serosurveillance studies in Kenya have indicated high mpox seropositivity rates in these groups in the absence of reported infections, indicating significant underreporting of urban mpox cases [23].

METHODS

Population Recruitment and Sample Collection

This cross-sectional study sought to determine the seroprevalence of mpox among key populations in two geographically distinct urban centers of DRC: Kinshasa (Kinshasa Province) and Goma (North Kivu Province). These locations were selected due to their high population density and mobility, factors that elevate the risk of MPXV transmission. Between April and June 2024, participants were recruited from three suspected at-risk sub-populations: self-identified MSM, self-identified SW, and other at-risk members of the general population based on their location and occupation. At the time of enrollment, mpox-specific vaccines were not available for administration.

In order to enroll these often socially subtle, at-risk groups, recruitment strategies relied on a combination of time-location (TLS) and snowball sampling. Peer educators (PEs), who were members of the MSM and SW communities, played a key role in the recruitment process [24]. In collaboration with the National Program for the Fight against HIV/AIDS and Sexually Transmitted Infections (French acronym, PNLS) and PEs, key locations frequented by these populations, such as health facilities, bars, nightclubs, markets and city entrances, were identified for sampling.

Eligible participants were recruited if they were ≥18 years old and self-identified as either a MSM or SW. Additionally, at PE-directed sampling sites, non-MSM and non-SW were enrolled as a convenience sample of an otherwise at-risk population (ARP); the ARP also included those participants recruited at farms and abattoirs (suspected occupational zoonotic exposure). All participants consented to both survey participation and blood sampling [24]. This study was approved by the Institutional Review Boards of the University of California, Los Angeles (IRB#23–000676), University of Manitoba (HS25837) and the Kinshasa School of Public Health, University of Kinshasa, DRC (ESP/CE/161/2024).

Data collection consisted of two main components: an interviewer-led semi-structured questionnaire, and serum collection at enrollment. The survey instrument, hosted on a secure survey platform server (*SurveyCTO* (Version 2.0), Dobility, Inc), included questions on

demographic characteristics (e.g. age, education, marital status), sexual and clinical history, knowledge of mpox transmission, and zoonotic and sexual risk factors. Additionally, trained phlebotomists collected a venous blood sample that was aliquoted in the field into serum, and plasma and buffy coat samples.

Serologic Testing

Serum samples were evaluated for reactivity using a four-plex immunoassay of three synthetic peptides derived from the right terminal region B of MPXV, B21R.179/180, B21R.185/186, and B22R.64/65 [25], as well as the highly-conserved *Poxviridae* peptide, H3L [26,27]. In brief, each peptide was first conjugated to bovine serum albumin before covalent attachment to magnetic beads with distinct fluorescent signatures (Luminex Corp, Austin, TX, USA). A master-mix bead solution containing Phosphate Buffered Saline (PBS) with 0.75 mol/NaCl, 5% heat-inactivated fetal bovine serum (Gibco-Invitrogen, France), 1% bovine serum albumin, and 0.2% Tween-20 (Sigma-Aldrich, France) was added to each well of 96-well flat-bottom chimney plates (Greiner Bio One, Germany). Diluted serum samples (1:200) were subsequently added to individual wells in duplicate; following washing steps, a secondary biotin-labeled anti-anti-human IgG antibody (BD-Pharmingen, France) was added. Finally, streptavidin-R-phycoerythrin (Fisher Scientific/Life Technologies, France) was introduced; with antigen-antibody binding quantified as median fluorescence intensity (MFI) per 100 beads using a Magpix instrument (Bio-Rad, France). All sample testing was completed on-site in Kinshasa, DRC.

As described by Kinganda-Lusamaki et al., the mean MFI plus three standard deviations of an 90-person mpox-negative panel was calculated as the respective seroreactivity threshold for each of the MPXV-specific peptides, B21R.179/180, B21R.185/186, and B22R.64/65 [18]. Given the unknown orthopoxvirus vaccination status of the 90-person panel, the cut-off value for anti-H3L reactivity was calculated using the likelihood ratio from a Receiver Operating Characteristic (ROC) curve (Supporting Information: Figure S1 of the original publication[18]).

Seroreactivity results were binarized, calculated by dividing the MFI value of each sample by the corresponding cut-off for each peptide; an index >1 was considered seroreactive. As positivity to any individual peptide of the four-plex was deemed sufficient for a seroreactive result [25], the maximum index value was considered for each sample.

Statistical Analysis

All data analysis was completed with SAS version 9.4 (SAS Institute, Cary, NC) and R version 4.3.3 (R Foundation for Statistical Computing, Vienna, Austria). Descriptive statistics were generated for participant demographics and seroreactivity rates. Unadjusted measures of association between categorical variables were assessed using chi-square tests, while continuous variable associations were evaluated with two-tailed Student's t-tests. Statistical significance was determined using p-values ($p < 0.05$), and 95% confidence intervals (CI) were reported to assess the precision of the estimates.

Given the spread of zoonotic and sexual risk factors and unadjusted associations with detected seroreactivity, a five-point composite score was generated for both zoonotic and sexual risk **(Figure 1)**. Variables included in these scores were selected based on completeness as well as *a priori* understandings of mpox transmission through either zoonotic or human-to-human routes. Zoonotic risk scores were calculated based on the binary responses to the following statements, with each "yes" response equating to one point on the risk scale: 1) Have you ever visited a forested/wooded area?; 2) Have you ever hunted wild animals?; 3) Have you ever prepared a wild animal for cooking?; 4) Have you ever consumed bushmeat?; and 5) Do you work with or interact with wild animals? Similarly, the sexual exposure risk score was calculated with the following responses: 1) Do you have a primary sexual partner? ("no" response as one point); 2) In the last three weeks have you had sexual relations with more than one person?; 3) Did you use a condom the last time you had sex? ("no" response as one point); 4) Have you ever had sex in exchange for money, drugs, food or accommodation?; and 5) a total partner count greater than

the median of the study population (as calculated by total estimated male and female partner counts).

To further understand the relative contributions of predictor variables in explaining seroreactivity results, the R packages "tree", "randomForest" and "party" [28] were used to generate classification trees and iterative regression models to identify top predictor variables and rankings of predictors contributing to explained seroreactivity (binary outcome, reactive = 1). A final model was employed that consisted of 500 trees; at each node, with 5 covariates randomly selected as splitting candidates for each tree constructed. Model fit was evaluated using an out-of-bag (OOB) error estimate and presentation of the class confusion matrix. Additionally, feature importance was calculated via a variable importance measure (mean decrease in Gini impurity, see Liaw and Wiener for details) and presented alongside a corresponding classification tree. To address class imbalance, an over-weighted model of 350 randomly selected participants including all 53 seroreactivity individuals was run; yet this only marginally improved the confusion matrix of the model. Lastly, logistic regression models to adjust for selected covariates were included to determine potential drivers of seroreactivity with Model 1 including selected demographic characteristics and the collapsed sexual risk score, and Model 2 the collapsed zoonotic risk score.

RESULTS

Overall Seroreactivity

A total of 1,823 participants were enrolled between April and June 2024— 913 (50.1%) from Kinshasa and 910 (49.9%) from Goma. Across specifically recruited sub-populations: 520 MSM (28.5%), 586 SW (32.1%), 110 MSM and SW (6.0%), and 607 ARP individuals (33.3%) were enrolled, with equivalent distributions in the two cities. Serological testing indicated an overall mpox seroprevalence of 2.9% (53/1823) among all participants, with 4.8% reactivity detected among ARP participants. In contrast, reactivity rates were lower among MSM (2.1%) and SW

(1.7%) and MSM/SW (2.7%) populations. Seroreactivity was similar across urban centers, with 3.1% detected in Kinshasa and 2.8% in Goma. By antigen, 22 participants were reactive to H3L (1.2%), 17 to B21R.185/186 (0.9%), 12 to B21R.179/180 (0.7%) and 5 to B22R.64/65 (0.3%). **(Table 1).** Of all 53 reactive individuals, only three respondents were considered reactive to more than one antigen, one reactive to H3L and B21R 180/186, and another two respondents to the both B21R antigens **(Supplementary Figure 1)**. No individual was reactive to all four antigens presented.

Seroreactivity by Population Demographics and Medical History

The median age of participants was 25 years (IR: 21-32), with the majority (46.8%) aged 18–24 years and decreasing representation in older age groups **(Table 2)**. Seroreactivity was significantly associated with age (p<0.0001). Participants aged ≥45 years demonstrated markedly higher seroprevalence (11.1–19.2%) compared to younger age groups (2.2–2.9%). When binarizing age by those born before or after the cessation of smallpox vaccination campaigns[29], this difference in seroreactivity is more pronounced with those born pre-1984, reporting 7.8% reactivity (p=0.0001). While the collapsed seroreactivity variable indicates higher reactivity among those born pre-1984 (n = 12/153) , by antigen, this is almost exclusively driven by H3L reactivity (n = 11, 91.7%) **(Supplementary Figure 1)**. While not statistically significant (p=0.413), heterosexual individuals had a higher overall proportion of seropositivity (3.3%). No significant difference in seroprevalence was observed based on recruitment site (e.g., bars, clubs, health facilities) (p=0.913) or among those reporting zoonotic exposures such as working at abattoirs (p=0.6914). Educational attainment was not significantly associated with seroprevalence (p=0.692), though individuals with only primary education had a slightly higher seroreactivity rate (5.3%) compared to those with secondary or university-level education (2.4–3.2%).

While no significant association was observed (p=0.247) between STI histories and seroreactivity, trends such as higher rates of seroreactivity among those respondents who had

no history of past STI diagnosis (3.5% vs 2.6%, respectively) were observed. Interestingly, among respondents with a self-reported history of STIs (n = 1136), those who had reported past syphilis diagnoses were more seroreactive (5/102, 4.9%) than respondents reporting other STIs. Notably, of the 25 individuals who self-identified as HIV-positive, no reactivity was detected.

Seroreactivity by Zoonotic and Sexual Exposures

Beyond specific demographic characteristics or medical histories, this study sought to evaluate seroreactivity rates by general zoonotic and sexual risk factors **(Table 3)**. Seroreactivity was higher among participants reporting selected wildlife or forest contacts: 22 participants of the 458 who reported ever visiting wooded or forested areas were MPXV seroreactive (4.8%; p=0.0052); 4.3% (22/516; p=0.031) of those who prepared or cooked wild animals, as well as 3.7% of participants who reported bushmeat consumption (37/991; p=0.0232). Occupational animal contact (working at abattoirs or farms, p=0.4595) and reported personal protective equipment (PPE) use were not significantly associated with seroreactivity.

Across all participants, there was a significant association between reporting past sex with both men and women and MPXV seroreactivity: participants who reported ever having had sex with a man had lower rates of reactivity (2.3%), when compared to those who had ever had sex with a woman (4.0%). No significant associations were observed between sexual behaviors such as condom use, group sex, and transactional sex, with seroreactivity.

The random forest classification model obtained an OOB error rate of 3.1% indicating strong predictive performance (n = 1705 complete observations). However, while the class 0 error of this model was 0.0012, the reciprocal class 1 error was 0.981, indicating poor sensitivity of the model to detect true seroreactive cases. Across both the feature importance assessment **(Figure 2**) and the generated classification tree **(Figure 3)**, the age group split of respondents younger or older than 45 years old was identified as the predominant covariate influencing the mpox

seroreactive outcome. While handling animal waste is presented as a second node of the classification tree, it is unclear as to why such exposure would have meaningfully contributed to predictive seroreactivity among a younger, urban population who participated in this study, and may simply be acting as a surrogate for more general behavioral/social conditions (i.e., it is possible that this specific question may be indicative of a more general health/hygiene discrepancy between participants or locations).

While the random forest approach incorporates potential confounders as splitting variables, this statistical method does not provide adjusted estimates for select risk factors and the outcome of binary seroreactivity. To address this, in two separate multivariate logistic regression models adjusting for sex and zoonotic risk, or population type, location, sex and age, age category was consistently identified as a significant factor associated with seroreactivity (Wald p-value = 0.0009, and 0.0021, **Table 4**). In Model 1, when adjusting for the collapsed sexual risk scores, participants aged 45-54 and 55+ had 3.92- and 7.77-times higher odds (CI 1.42-10.81; 2.37-25.52) of seroreactivity than those participants aged 18-24. Additionally, this trend of increased odds in older populations was maintained when adjusting collapsed zoonotic risk scores and other demographic confounders in Model 2: participants aged 45-54 and 55+ had 3.49- and 6.11-times higher odds than those participants aged 18-24 (CI: 1.30-9.37; 1.86-20.03).

DISCUSSION

This baseline serosurvey of high-risk urban populations provides a unique cross-sectional estimate of mpox exposure rates prior to the sustained human-to-human transmission of the virus in Kinshasa and Goma first reported in the later part of 2024. While overall seroprevalence was low (2.9%), patterns across demographic and behavioral groups offer important insights into evolving transmission dynamics.

The sample collection period of this study suggests that the detected infections likely resulted from sporadic zoonotic introductions and small, self-limiting clusters--similar to those historically documented in Central Africa--rather than the prolonged human-to-human transmission chains now observed in urban centers of DRC [30]. These initial transmission dynamics may be best described as stuttering chains with an initial spillover event seeding weak human-to-human spread [31-33]. Specifically, in Kinshasa, the first case of Clade Ib MPXV was detected in July 2024 and large-scale detection of sustained human transmission was not confirmed until mid-August 2024, well after the sampling period of this study [30,34]. Similarly, the first cases of Clade Ib MPXV were not confirmed in Goma until June 2024, at the same time that this study was conducted[35]. Given the general timeline of antibody development, it is highly likely that all detected seroreactivity predates the confirmed introduction of Clade Ib MPXV in both cities. While mpox in endemic regions has been traditionally attributed to rural wildlife-to-human spillover, our findings show that such introductions may also occur in urban contexts, potentially seeding emerging transmission networks.

Seroprevalence varied across demographic groups, with older age strongly associated with seroreactivity. However, the higher prevalence among older participants could reflect cumulative lifetime exposure or residual immunity from historic smallpox vaccination campaigns. Moreover, when dividing the cohort by those born before and after 1984 (presumed vaccination), all but one of the 12 individuals identified as reactive were specifically reactive to the H3L antigen. While smallpox was officially declared as eradicated in 1980, there has been evidence of continued sporadic vaccination campaigns in the DRC up until 1984[29]. Here, we specifically chose birth year of 1984 as a more conservative cut off for potential exposure to the vaccina-based vaccine. Notably, the age split at age 45 as indicated by the regression tree aligns with a 1980 birth year cut point, potentially indicating that exposure among this group (the same 12 reactive

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individuals as identified by the 1984 cut point) is instead driven by historic orthopoxvirus vaccination.

The H3L envelope protein is a well conserved region across the *Poxviridae* family, including vaccinia virus (VACV) [27]. As this antigenic target was included as a component of the binary reactivity status, a history of VACV exposure—whether via vaccination or natural infection—may instead be driving this observed association. This potential cross-reactivity with vaccinia-derived immunity should not be understated as the potential true mechanism driving higher reactivity rates among older participants. However, as we did not specifically collect vaccinia vaccination history, respondent age serves as a proxy for this exposure where those over 45 years old are highly likely to have been vaccinated as part of smallpox eradication practices.

Yet, among a separate cohort of individuals with differential orthopoxvirus exposures, only 6/26 smallpox vaccinees were reactive to H3L (unpublished data). Previous studies have indicated that waning smallpox immunity in DRC has gradually eroded cross-protective immunity, thereby increasing the proportion of immunologically naïve individuals susceptible to mpox [5]. As the younger MSM and SW of this cohort are unlikely to have been vaccinated for smallpox, these populations are likely to suffer additional disease burden as human-to-human mpox transmission persists. The lower seroreactivity rates, and by extension, immune-naive status among MSM and SW identify MSM and SW populations as high priority groups for mpox vaccination campaigns.

The highest seroreactivity was detected among the sampled ARP (farm workers, non-MSM and non-SW) (4.8%), a pattern consistent with traditional pathways of mpox exposure via animal-to-human contact. Although age was identified as the most predominant predictor of seroreactivity—both by the random forest and generalized linear regression model approaches—notable associations were also observed with self-reported behaviors indicative of zoonotic risks: specifically, bushmeat consumption and visiting forested areas. Interestingly, these activities are

typically considered to be elements of rural exposure profiles; yet over 50% of respondents in Kinshasa and Goma had reported consuming wild animals at least once in their lifetime. This finding suggests that the boundaries between rural and urban exposure routes may be increasingly blurred, emphasizing the interconnected nature of zoonotic risk in the urban landscape of the DRC. Future investigations should build off these findings to better define which key factors may be driving this zoonotic risk among this ARP, as well as sampling of a true urban general population to establish a more universal urban seroreactivity threshold irrespective of sub-population by occupational or behavioral classifications. Furthermore, the sampling design of this approach further limits generalizability of these seroreactivity rates to the larger urban population; these results are limited in their applicability beyond those specific cohorts recruited for this study.

Unlike similar studies conducted in Kenya which identified strong seropositivity among SW and MSM communities in Nairobi [23], sexual risk behaviors and identification as SW or MSM were not identified as drivers of mpox seroreactivity in Kinshasa and Goma. However, these differences may be attributed to the sampling type – prospective as compared to retrospective from convenience sampling – antibody targets of the serologic assay, and other additional variables including community-specific behavioral differences. Statistical associations were only observed between reactivity and reported lifetime partner counts and frequent intimate contacts, yet these exposures did not supersede age as drivers of the reactivity outcome when assessed by the classification model. Sexual behavior patterns suggest that mixed sexual networks may facilitate early mpox spread. Heterosexual men and MSM reporting vaginal intercourse showed higher seroprevalence, indicating potential bridging between heterosexual and MSM networks. This differs from the 2022 Clade IIb global outbreak, which was largely confined to MSM populations [9].

While our iterative regression model achieved a low average error estimate, ultimately this approach had poor sensitivity in detecting seroreactive cases, most likely due to class imbalance. Such machine-learning approaches (e.g. partitioned tree classifications, random forests) can

always be improved with the inclusion of additional variables and response data; ongoing work is currently being conducted to re-enroll these same target populations to capture additional predictor variables and reassess seroreactivity.

As with any cross-sectional study design, this approach limits the ability to differentiate between recent and historic mpox exposure; lifetime exposure questions may also be subject to recall and reporting biases, leading to a possible over- or under-representation of the true exposure histories of participants. Future longitudinal sampling of these vulnerable urban populations, especially following the sustained human-to-human transmission reported in late 2024, would help to further elucidate which specific behavioral, sexual and zoonotic risks may be contributing to mpox spread in urban areas the DRC. Another limitation of this study is the binary approach to seroreactivity, while the B21R and B22R antigenic targets are MPXV-specific, the inclusion of H3L reactivity as a component of the MPXV reactivity definition may overestimate exposure. While supplementary data has demonstrated most reactivity (50/53 respondents) was to one antigen of the panel, further antigen-specific analysis should be conducted to better elucidate potential MPXV-specific exposure.

Our results suggest low-level baseline exposure to mpox among a subset of vulnerable urban populations. Of note, the four-plex MPXV serologic assay employed in this study has reported good sensitivity and high specificity to detect antibodies both early in infection, but also showed antibody reactivity decline overtime, up to two years after infection [18,36]. This facet of the assay functionality is in line with our conclusions that those detected seroreactive individuals may have higher reactivity rates with age—whether that be historic orthopoxvirus vaccination status, or more simply increased exposure frequency over time as an inhabitant of an mpox-endemic region. Ultimately, these results define a baseline exposure rate to mpox, specifically among populations suspected to be at higher risk in an urban setting.

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As Clade I MPXV continues to rapidly evolve, with increasing evidence of sustained human to human transmission of both Clades Ia and Ib [30], these results can inform context-specific behavioral and demographic vulnerabilities to guide early detection and public health intervention. While some transmission characteristics may be environmentally, behaviorally, or culturally bespoke, the MPXV transmission cycle itself is not unique to DRC, and by identifying particularly vulnerable populations in one setting, results are likely to help identify similar at-risk populations in others. Baseline serosurveys such as this are crucial for understanding the foundations of emerging epidemics. By characterizing pre-outbreak exposures and the complex interplay of zoonotic and behavioral risk factors among key populations in an endemic setting, this work provides a vital reference point for interpreting current and future urban mpox outbreaks—both in DRC, and abroad.

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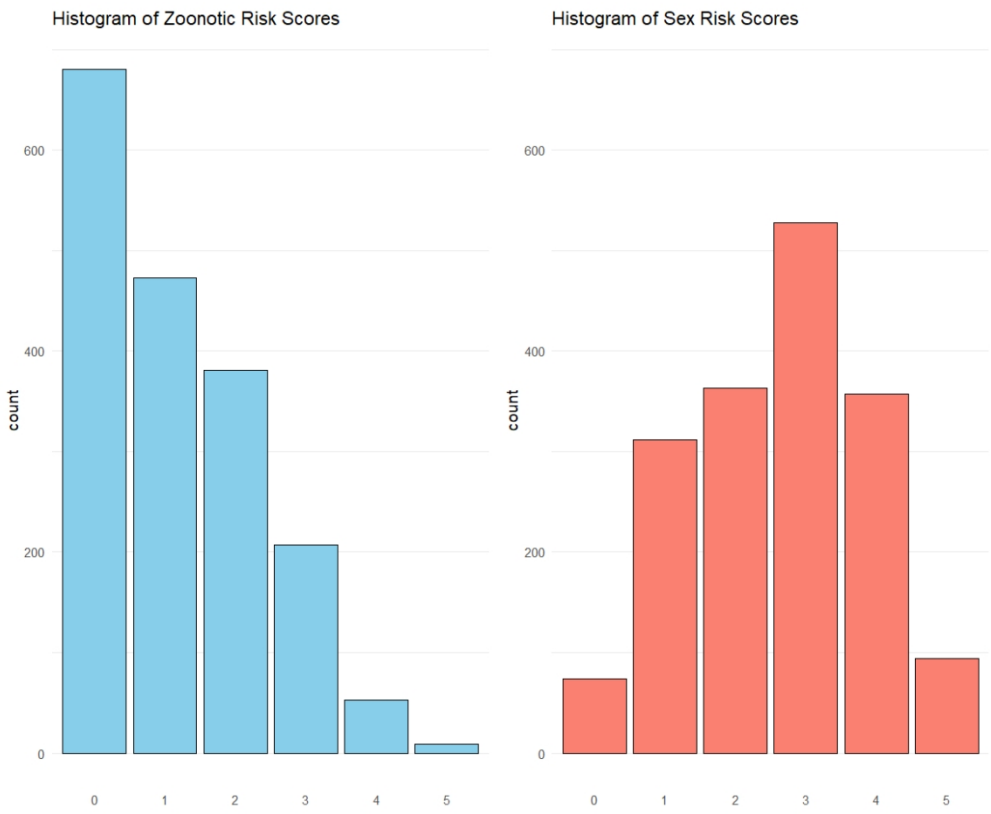

Figure 1. Histograms of Zoonotic (n = 1803) and Sex Risk Scores (n = 1728). See Supplementary Figures for stratification by age, location, sex and population of interest.

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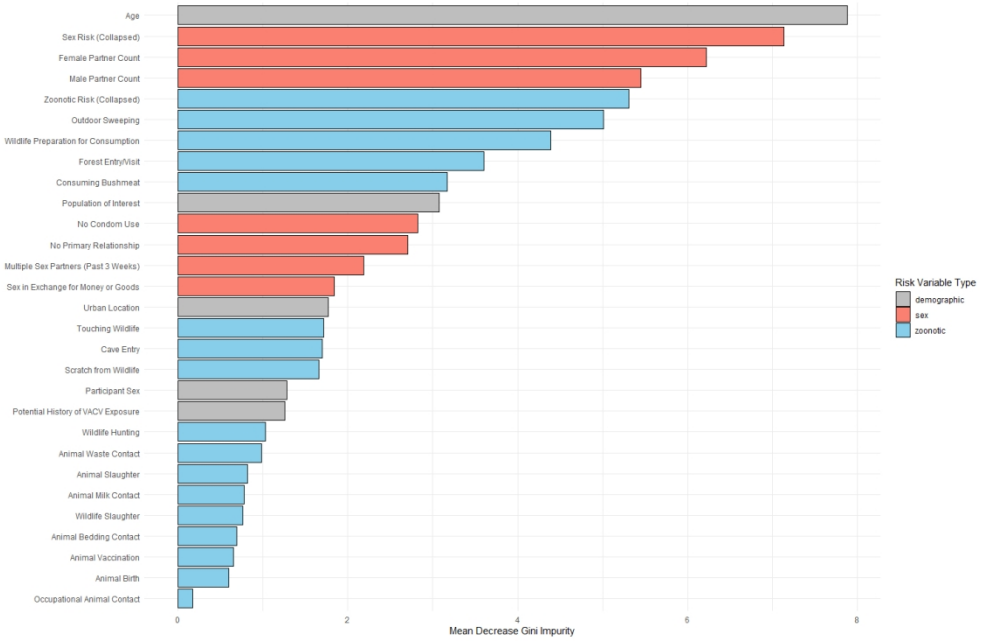


Figure 2. Age as a Key Determinant of Seroreactivity: Feature Importance as generated by Random Forest Model. Feature importance is evaluated as the mean decrease in Gini impurity of each variable in aggregated across all trees of the iterative Random Forest model. Greatest reduction in mean Gini impurity equates to the greatest relative significance of a variable within the context of this model; participant age is ranked as the most influential variable.

998x641mm (38 x 38 DPI)

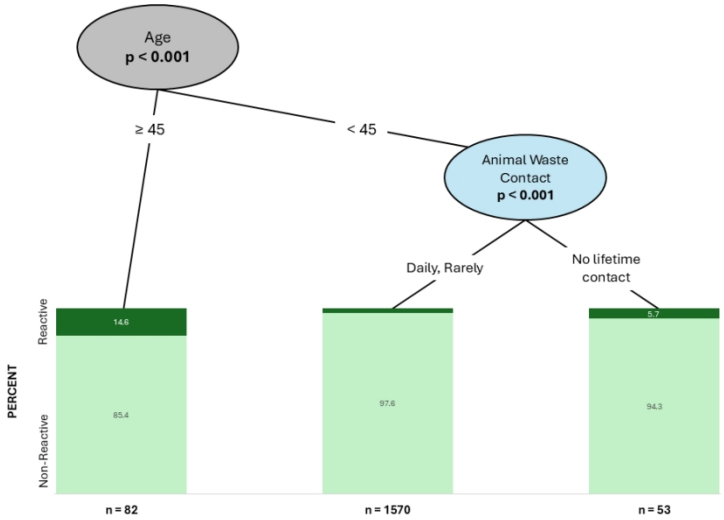


Figure 3. Classification Tree Indicates Age as the Primary Determinant of Seroreactivity. The classification tree plot indicates age as the most informative predictor of seroreactivity with the first significant split ($p < 0.001$) at < 45 and ≥45 years old; among the older participants, 14.6% of this node were seroreactive to MPXV.

855x481mm (38 x 38 DPI)

Population Type, Location	Total (%)	Detected Seroreactivity H3L		B21R 179/180		B21R 180/186		B22R 64/65		Sum Reactivity	
All Participants	**1823**	22	1.2	12	0.7	17	0.9	5	0.3	**53**	**2.9**
Population of Interest											
Men who have sex with Men (MSM)	**520** (28.5)	3	0.6	3	0.5	4	0.7	2	0.4	**11**	**2.1**
Sex Worker (primary or secondary occupation, SW)	**586** (32.1)	4	0.7	3	0.5	3	0.5	1	0.2	**10**	**1.7**
MSM and Sex Worker	**110** (6.0)	1	0.9	0	0.0	2	1.8	0	0.0	**3**	**2.7**
At-Risk Population (ARP)	**607** (33.3)	14	2.3	6	1.0	8	1.3	2	0.3	**29**	**4.8**
Urban Center											
Kinshasa	**913** (50.1)	13	1.4	5	0.6	11	1.2	1	0.1	**28**	**3.1**
Goma	**910** (49.9)	9	1.0	7	0.8	6	0.7	4	0.4	**25**	**2.8**

[1]Reported as n, % unless otherwise noted.

Demographics and Medical History	All N = 1823		Seroreactive n = 53 (2.9)				Chi Sq p-value
	N	Col Pct	N	Row Pct	Lower 95% CI	Upper 95% CI	
Population of Interest							
At-Risk Population (ARP)	607	33.3	29	4.8	3.3	6.8	0.0086
Men who have sex with Men (MSM)	520	28.5	11	2.1	1.2	3.7	
Sex Worker (primary or secondary occupation, SW)	586	32.1	10	1.7	0.9	3.1	
MSM and Sex Worker	110	6.0	3	2.7	0.9	7.7	
Urban Center							
Kinshasa	913	50.1	25	2.8	1.9	4.0	0.6847
Goma	910	49.9	28	3.1	2.1	4.4	
Age Category							
18-24	853	46.8	20	2.3	1.5	3.6	<0.0001
25-34	636	34.9	14	2.2	1.3	3.7	
35-44	245	13.4	7	2.9	1.4	5.8	
45-54	63	3.5	7	11.1	5.5	21.2	
55+	26	1.4	5	19.2	8.5	37.9	
Age Binary pre-/post-Smallpox Vaccination Cessation							
Born after 1984 (assumed no smallpox vaccine)	1670	91.6	41	2.5	1.8	3.3	0.0001
Born during or prior to 1984	153	8.4	12	7.8	4.5	13.2	
Sex at Birth							
Male	1010	55.4	34	3.4	2.4	4.7	0.1935
Female	813	44.6	19	2.3	1.5	3.6	
Sexual Orientation							
Straight	1214	66.6	40	3.3	2.4	4.5	0.4129
Gay	371	20.4	6	1.6	0.7	3.5	
Bisexual	273	15.0	7	3.0	1.2	5.2	
Neutral	1	0.1	0	0.0	0.0	79.3	
Site of Recruitment							
Commercial Farm	3	0.2	0	0.0	0.0	56.1	0.9134
Abbatoir	44	2.4	2	4.6	1.3	15.1	
Bar/Club	559	30.7	14	2.5	1.5	4.2	
Private Home	98	5.4	4	4.1	1.6	10.0	
Health Facility	303	16.6	10	3.3	1.8	6.0	
Other/INRB Goma	816	44.8	23	2.8	1.9	4.2	
Education Level							
None	43	2.4	0	0.0	0.0	8.2	0.6914
Started Primary School	75	4.1	4	5.3	2.1	12.9	
Finished Primary School	572	31.4	16	2.8	1.7	4.5	
Finished Secondary School	713	39.1	23	3.2	2.2	4.8	
Apprentice	14	0.8	0	0.0	0.0	21.5	
College/University	337	18.5	8	2.4	1.2	4.6	
Graduate school	69	3.8	2	2.9	0.8	10.0	
HIV Status							
Yes	25	1.4	0	0.0	0.0	13.3	0.3840
No	1798	98.6	53	3.0	2.3	3.8	
History of STI Diagnoses							
Past STI Diagnosis	1136	62.3	29	2.6	1.8	3.6	0.2470
No Past STI Diagnosis	687	37.7	24	3.5	2.4	5.1	
Among those with a history of STI Diagnosis...	n = 1136 (62.3)						
Did you have a rash/lesions?							
No	343	30.2	9	2.6	1.4	4.9	0.8550
Yes- a rash with skin lesions	550	48.4	15	2.7	1.7	4.5	
Yes- a rash without skin lesions	243	21.4	5	2.0	0.9	4.7	
Specific Diagnosis							
Gonorrhoea	312	27.5	6	1.9	0.9	4.1	0.4080
Chlamydia	47	4.1	0	0.0	0.0	7.6	0.2570
Syphilis	102	9.0	5	4.9	2.1	11.0	0.1150
Anal or genital herpes	461	40.6	9	2.0	1.0	3.7	0.2890
Other	373	32.8	10	3.7	1.5	4.9	0.8480
Unknown	97	8.5	2	2.1	0.6	7.2	0.7490

Table 2. MPXV Seroreactivity by Sample Population Characteristic. Chi-square p-values less than 0.05 are highlighted in green.

	N MISSING	All N = 1823 N	All N = 1823 ColPct	Seroreactive n = 53 (2.9) N	Seroreactive n = 53 (2.9) ColPct	Seroreactive n = 53 (2.9) RowPct	Chi Sq P-value
Reported Zoonotic Exposure							
Have you ever done any of the following activities, in your life?							
Enter a cave or mine	1	208	11.4	8	15.1	3.9	0.3928
* Visit wooded/forested areas		458	25.1	22	41.5	4.8	0.0052
* Hunt wild animals	1	67	3.7	3	5.7	4.5	0.4363
Skinned/slaughtered/ or butchered a wild animals	2	105	5.7	3	5.7	2.9	0.9733
* Prepared as in cooking a wild animal	3	516	28.3	22	41.5	4.3	0.0310
Play with or get scratched or bitten by any wild animals	2	120	6.6	6	11.3	5	0.1589
Touched a wild animal that was already dead	2	124	6.8	5	9.4	4	0.4414
* Eat Bushmeat	5	991	54.5	37	69.8	3.7	0.0232
Sweep inside or around your home	4	862	47.4	26	49.1	3	0.8051
* Do you work with or interact with animals (wild or domestic) as a part of your occupation?							
Yes	13	96	5.3	4	7.6	4.2	0.4595
No		1714	94.7	49	92.5	2.9	
Reported Sexual Exposure							
Have you ever had sex with a man?							
Men - Yes	1	1409	77.3	32	60.4	2.3	0.0028
Men - No		413	22.7	21	39.6	5.1	
* How many male partners do you estimate you have had in total - in your life?							
None		413	22.7	21	39.6	5.1	
Less than 10		483	26.5	14	26.4	2.9	
10 to 20	1	164	9.0	8	15.1	4.9	0.0027
21 to 50		161	8.8	3	5.7	1.9	
More than 50		601	33.0	7	13.2	1.2	
Have you ever had sex with a woman?							
Women - Yes	4	728	39.9	29	54.7	4.0	0.0267
Women - No		1091	60	24	45.3	2.2	
* How many female partners do you estimate you have had in total - in your life?							
None		1091	59.8	24	45.3	2.2	
Less than 10		433	23.8	10	18.9	2.3	
10 to 20	4	138	7.6	10	18.9	7.3	0.0026
21 to 50		78	4.3	5	9.4	6.4	
More than 50		79	4.3	4	7.6	5.1	
* The last time that you had sex, did you use a condom?							
Yes	67	839	47.8	22	42.3	2.6	0.4227
No		917	52.2	30	57.6	3.2	
* Do you currently have a primary sexual partner (e.g. boyfriend, girlfriend, husband or wife)?							
Yes	65	1215	69.1	34	65.4	2.8	0.5548
No		543	30.9	18	34.6	3.3	
* Have you ever had sex with someone in exchange for money, drugs, food or accommodation?							
Yes	59	1187	67.3	29	55.8	2.4	0.0723
No		577	32.7	23	44.2	4	
In the past 6 months, what has been the the sex/gender of your sexual partners? Select all that apply.							
Women		605	34.2	24	46.2	4	0.0647
Men		1340	75.7	29	55.8	2.2	0.0007
Other	53	9	0.5	1	1.9	11.1	0.1455
Prefer not to answer		1	0.1	0	0	0	0.8618
I have not had any sexual relations in the past 6 months		71	4	4	7.7	5.6	0.1697
* In the last three weeks, have you had sexual relations or intimate contact with more than one person?							
Yes	76	1034	59.2	23	44.2	2.2	0.0259
No		713	40.8	29	55.8	4.1	
Were any of those sexual or intimate contacts with more than one person at the same time?		n = 1034 (51.6)					
Yes	169	446	51.6	5	29.4	1.1	0.065
No		419	48.4	12	70.6	2.9	

		aOR	Lower CI	Upper CI	WALD p-value
MODEL 1 SEX RISK reactive(x) = age_cat + sex + loc + popcohort + sex_risk	**n = 1728**				
Age					
	18-24	REF			
	25-34	0.866	0.426	1.762	0.6911
	35-44	1.091	0.437	2.726	0.8513
	45-54	**3.923**	**1.423**	**10.818**	**0.0083**
	55+	**7.774**	**2.368**	**25.521**	**0.0007**
Sex					
	Male	REF			
	Female	0.714	0.296	1.725	0.454
Urban Center					
	Kinshasa	REF			
	Goma	1.158	0.624	2.149	0.6418
Population Cohort					
	At Risk Population	REF			
	MSM	0.451	0.192	1.060	0.0677
	MSM and Sex Worker	0.638	0.163	2.505	0.5195
	Sex Worker	0.500	0.158	1.587	0.2396
5-Point Sex Risk (Multiple partners in three weeks, QPQ Sex, Total Partner count > median, no condom use, non-monogamous/no primary partner)					
	0	REF			
	1	0.326	0.105	1.010	0.0520
	2	0.677	0.241	1.898	0.4580
	3	0.542	0.175	1.672	0.2863
	4	0.623	0.177	2.196	0.4620
	5	0.593	0.102	3.464	0.5619
MODEL 2 ZOO RISK model reactive(x) = age_cat + sex + loc + popcohort + zoo_risk	**n = 1803**				
Age					
	18-24	REF			
	25-34	0.773	0.381	1.568	0.4761
	35-44	0.964	0.389	2.390	0.9375
	45-54	**3.493**	**1.303**	**9.366**	**0.0129**
	55+	**6.109**	**1.863**	**20.025**	**0.0028**
Sex					

	Male	REF			
	Female	0.659	0.282	1.537	0.3341
Urban Center					
	Kinshasa	REF			
	Goma	0.96	0.518	1.778	0.8961
Population Cohort					
	At Risk Population	REF			
	MSM	0.491	0.223	1.085	0.0788
	MSM and Sex Worker	0.63	0.169	2.356	0.4925
	Sex Worker	0.585	0.225	1.519	0.2709
5-point Zoonotic Risk (working with animals, entering forest, preparing wild meat, eating bushmeat and hunting wild animals)					
	0	REF			
	1	1.691	0.697	4.101	0.2454
	2	**3.973**	**1.761**	**8.963**	**0.0009**
	3	**3.316**	**1.261**	**8.724**	**0.0151**
	4	2.357	0.467	11.91	0.2994
	5	<0.001	<0.001	>999.999	0.988

Table 4. Adjusted Odds Ratios of Selected Demographic Covariates and Sexual (Model 1) and Zoonotic (Model 2) Risk Scores. Wald p-values less than 0.05 are identified in bold, as are those point estimates considered significant.

Pairwise co-reactivity

Off-diagonal: n (% of total population) · Diagonal: individual reactive count

	H3L	B21R 179/180	B21R 180/186	B22R 64/65
H3L	22	0 (0.0%)	1 (0.1%)	0 (0.0%)
B21R 179/180	0 (0.0%)	12	2 (0.1%)	0 (0.0%)
B21R 180/186	1 (0.1%)	2 (0.1%)	17	0 (0.0%)
B22R 64/65	0 (0.0%)	0 (0.0%)	0 (0.0%)	5

Supplementary Table 1A. Co-Reactivity to all four antigens of the multiplex immunoassay among all cohort members (n = 1823).

Pairwise co-reactivity among respondents born pre-1984, potential VACV exposure

Off-diagonal: n (% of total population) · Diagonal: individual reactive count

	H3L	B21R 179/180	B21R 180/186	B22R 64/65
H3L	11	0 (0.0%)	0 (0.0%)	0 (0.0%)
B21R 179/180	0 (0.0%)	1	0 (0.0%)	0 (0.0%)
B21R 180/186	0 (0.0%)	0 (0.0%)	0	0 (0.0%)
B22R 64/65	0 (0.0%)	0 (0.0%)	0 (0.0%)	0

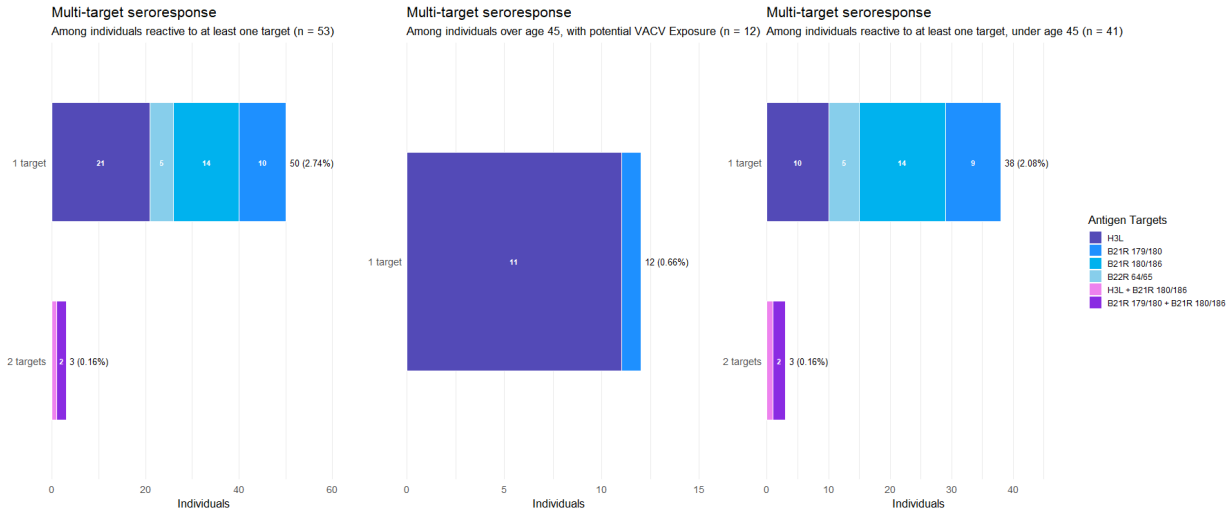
Supplemental Table 1B. With age as a proxy for smallpox vaccine exposure (VACV), Co-reactivity of all four antigens among cohort members born before 1984 (n = 153)

Pairwise co-reactivity among respondents born post-1984

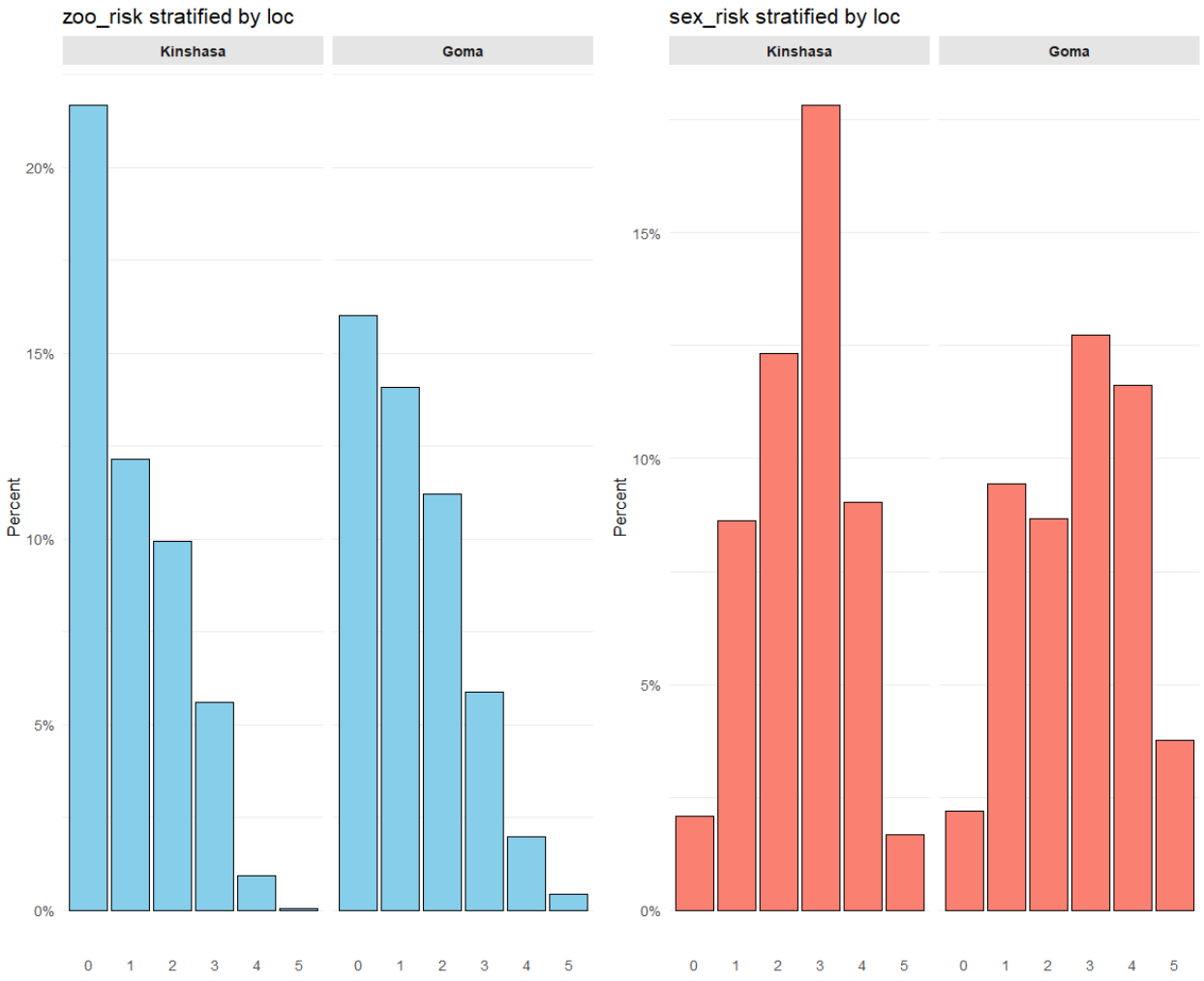
Off-diagonal: n (% of total population) · Diagonal: individual reactive count

	H3L	B21R 179/180	B21R 180/186	B22R 64/65
H3L	11	0 (0.0%)	1 (0.1%)	0 (0.0%)
B21R 179/180	0 (0.0%)	11	2 (0.1%)	0 (0.0%)
B21R 180/186	1 (0.1%)	2 (0.1%)	17	0 (0.0%)
B22R 64/65	0 (0.0%)	0 (0.0%)	0 (0.0%)	5

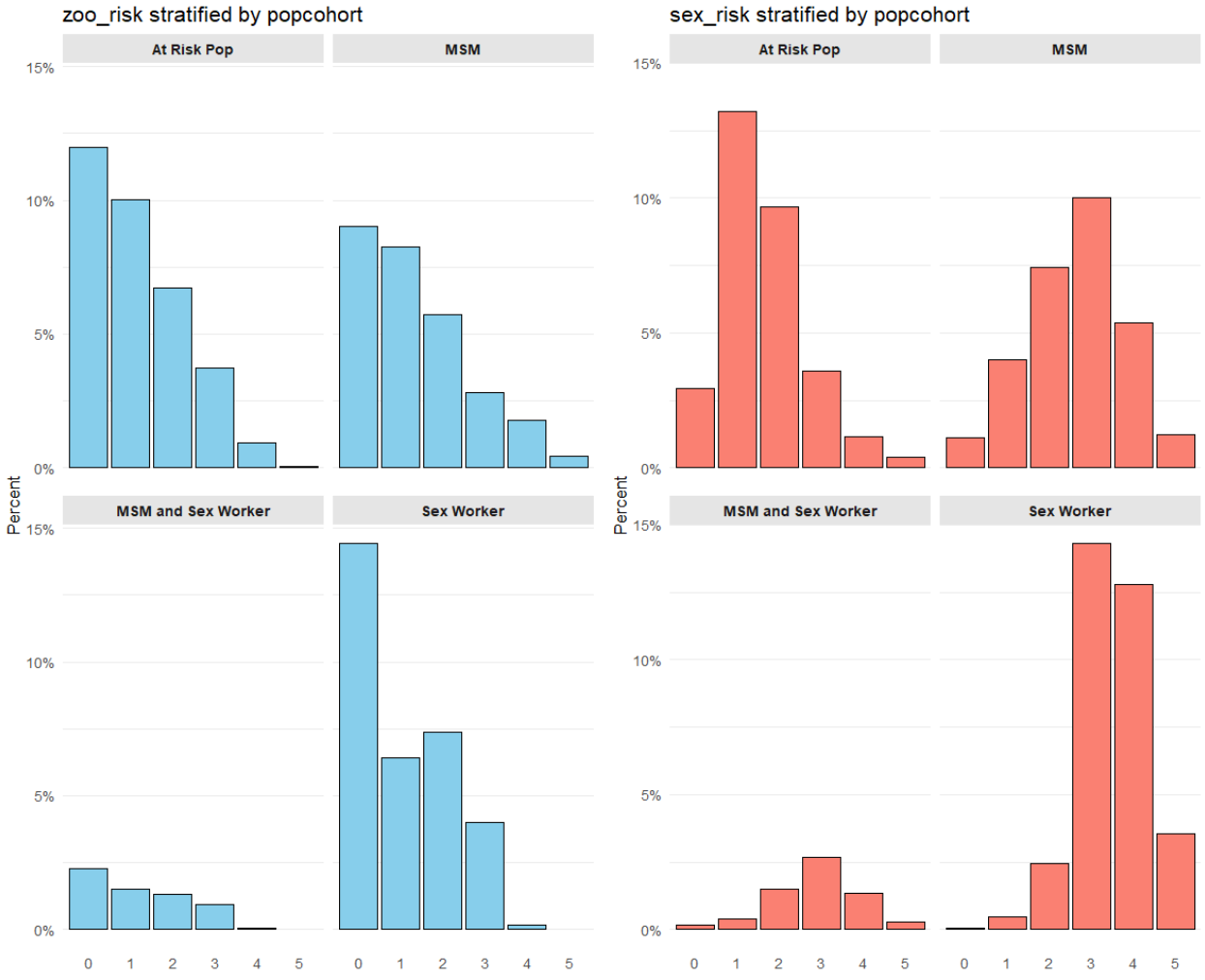
Supplementary Table 1C. Co-Reactivity to all four antigens of the multiplex immunoassay among cohort members born after 1984 (n = 1670).



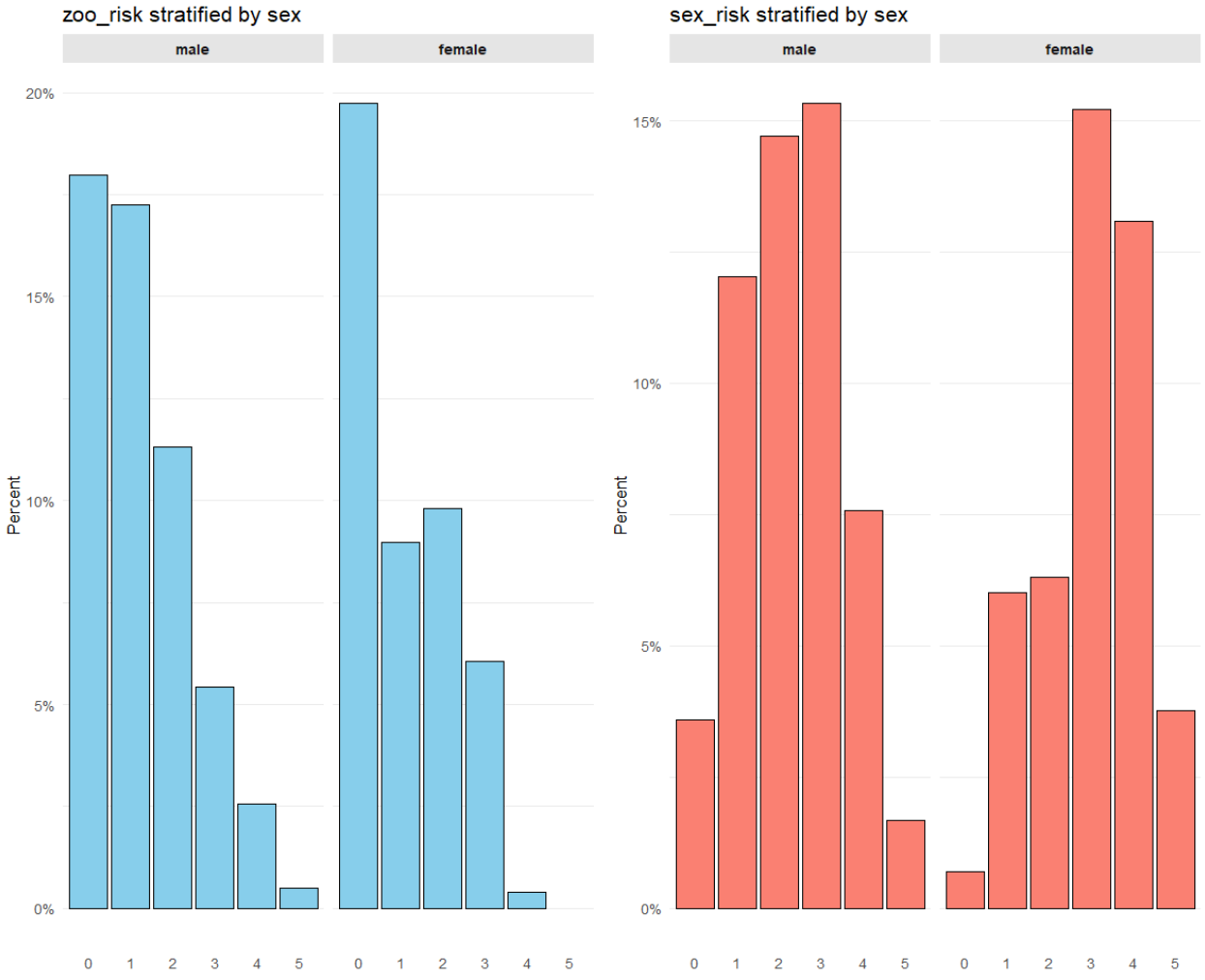
Supplemental Figure 1. Multi-Target Seroreactivity among all cohort members (n = 53), those born pre=1984 (n = 12) and those born post-1984 (n = 41)



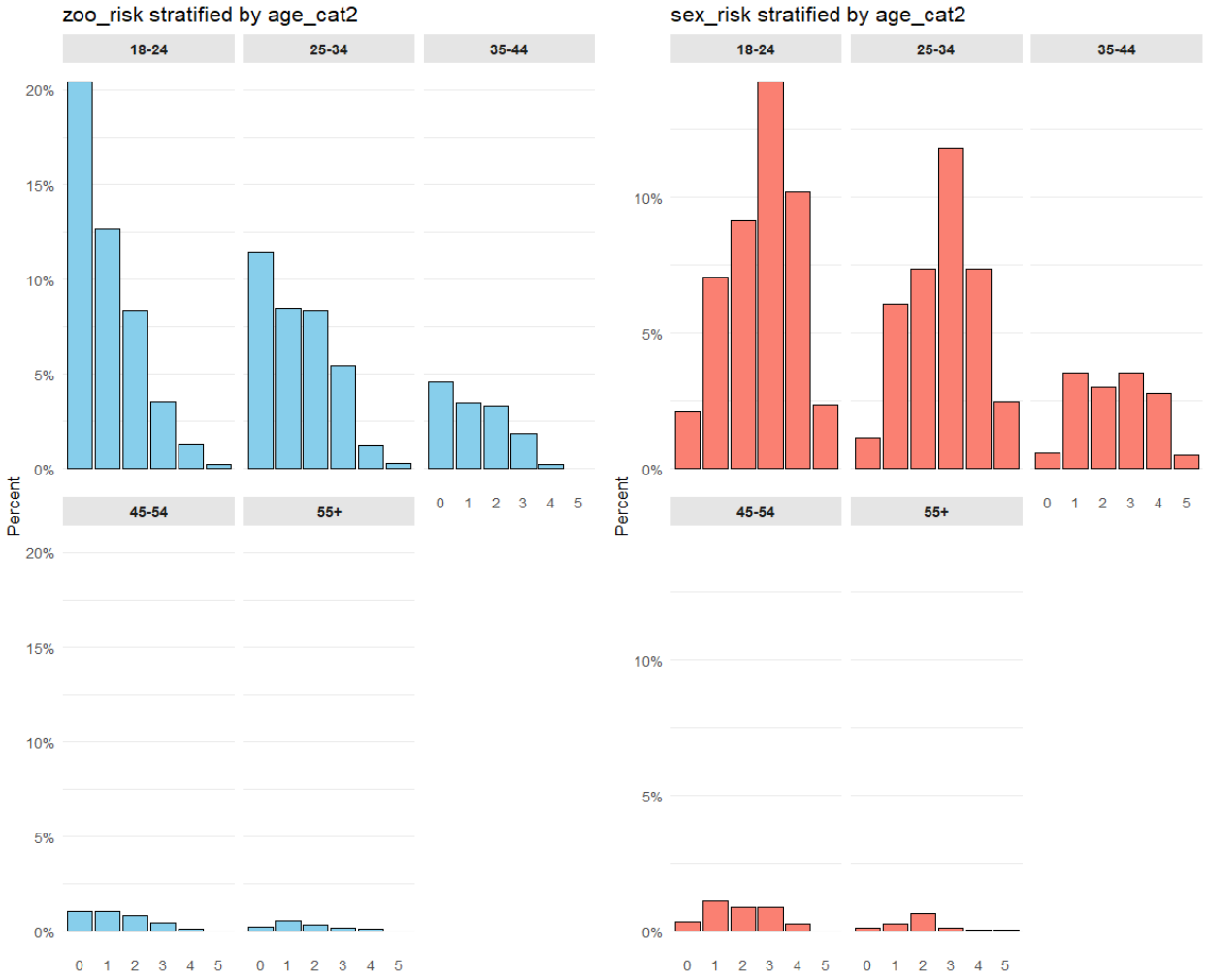
Supplemental Figure 2A. Zoonotic and Sexual Risk scores stratified by urban center.



Supplemental Figure 2B. Zoonotic and Sexual Risk scores stratified by population of interest.



Supplemental Figure 2C. Zoonotic and Sexual Risk scores stratified by respondent sex at birth.



Supplemental Figure 2D. Zoonotic and Sexual Risk scores stratified by age category.