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Shining light on the dark matter of pertussis: evidence for an asymptomatic carriage state from a longitudinal cohort of mother/infant dyads

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eLife Assessment

The authors describe the results of a longitudinal study of pertussis infection in mother/infant dyads in Lusaka, Zambia. This work represents an **important** contribution to our understanding of the global burden of pertussis. The evidence presented is **compelling**, with strengths including routine sampling irrespective of symptoms and rigorous qPCR methodology. The study is a significant and much-needed contribution, which sheds light on an often-overlooked dimension of pertussis transmission and opens avenues for future research and policy consideration.

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Abstract

Pertussis remains an enigma partly due to the uncertain impact of asymptomatic cases on transmission. Contributing to this knowledge gap is a lack of high-quality disease surveillance, particularly in those low- and middle-income countries that experience high disease burdens. Here we present analyses based on our prospective longitudinal surveillance of a rolling cohort of 1,315 mothers and their newborn infants in Lusaka, Zambia across 2015 (8,704 unique study visits). We detail the timing, duration, and intensity of qPCR-based IS481 signals in individual subjects and within mother/infant dyads. We find that IS481 signal strength: A) in mothers predicts contemporaneous and future IS481 detections in infants, B) in infants predicts, to a lesser extent, detections in mothers, and C) predicts respiratory symptoms in infants but not mothers. We profile a subgroup of 50 infants and 54 mothers who displayed evidence of persistent colonization (median duration 8 weeks) wherein most mothers were entirely asymptomatic. We also include a critical assessment of qPCR test reliability across IS481 signal strengths. Our results demonstrate the routine occurrence of long-duration, mild and minimally symptomatic pertussis infections and suggest that pertussis transmission occurs between minimally symptomatic mothers and their newborn infants.

Introduction

The resurgence of *Bordetella pertussis* in a number of countries with high routine immunization coverage (1) has called into question key assumptions about the transmission, immunology, and evolution of this highly contagious respiratory pathogen (2, 3, 4, 5, 1). Chief among these questions is the epidemiological importance of mild and asymptomatic pertussis infections (6, 7, 8, 9, 10).

Indeed, despite widespread recognition that mild pertussis is common (2), especially amongst adults (11, 12), its contribution to overall disease transmission and persistence remains poorly characterized (13, 6, 14).

Mild and asymptomatic pertussis has sometimes been considered the “dark matter” of pertussis transmission, owing to its elusive yet potentially significant impacts (9, 15). Prior to modern molecular surveillance, reliable detection of mild pertussis infections was challenging (16, 17, 18) and, in the pre-vaccine era, the epidemiological role of mild pertussis was largely ignored (19, 20, 21). As vaccination rates rose worldwide across the 20th Century, pertussis morbidity and mortality dropped sharply (22, 23, 24, 4). Yet local pertussis elimination has rarely been achieved, even in highly-vaccinated populations, unlike many similar childhood diseases such as measles, mumps, and rubella. Furthermore, the relative protection afforded by vaccination against transmissible infection versus carriage remains a subject of active debate, as does the precise epidemiological role of mild and asymptomatic pertussis infections (1). Consequently, understanding the determinants of pertussis transmission and persistence remains a pressing global health concern and an area of active public health research (25).

In recent decades, novel molecular surveillance tools have yielded remarkable insights into global pertussis dynamics, from phylodynamics (3, 26) to pathogen evolution (27, 28, 29, 30). Notably, qPCR-based testing for pertussis can detect mild pertussis much more reliably than previous culture-based methods (31, 32, 33). The most common of these tests targets a repetitive genetic sequence, IS481, that occurs at copy numbers in excess of 200 in *B. pertussis*. While IS481’s high copy number allows for high qPCR test sensitivity (<1 bacterium per reaction well), the interpretation of weak qPCR signals (i.e., low target counts) has been the subject of considerable debate. Some have argued for using additional tests (i.e., culture or serology) to ‘confirm’ qPCR results (34). Yet the discriminatory value of additional testing is far from clear at present given the low sensitivity of culture and the differing time scales of serology-based tests (35, 36, 34, 37). Furthermore, available tests struggle to reliably distinguish between *B. pertussis* presence, persistent colonization, and transmissible infection (38, 39). In this context, we view qPCR results as highly informative on their own, especially in the context of population and longitudinal surveillance.

Deployed at scale, qPCR offers the potential for a deeper understanding of when and where mild pertussis is most likely to occur, and how these infections contribute to local and regional disease dynamics. At present, however, high-income countries are responsible for the bulk of routine molecular pertussis surveillance (15). As these countries typically also experience the lowest burdens of most infectious diseases, such concentrated surveillance means that the best-studied populations may offer hard-won yet modest epidemiological insights.

Our 2015 Southern Africa Mother/Infant Pertussis study (SAMIPS) sought to bridge this gap through the use of targeted, high-resolution pertussis surveillance in a low-resource urban community (40, 41). Conducted in Lusaka, Zambia across one calendar year, SAMIPS followed a cohort of mothers and their young infants across these infants’ first 3-4 months of life. Each mother/infant dyad attended regularly-scheduled visits at a local public health clinic (PHC), where self-reported respiratory symptoms were recorded and nasopharyngeal (NP) samples were collected. At these visits, routine vaccinations and well-child care were also administered and recorded. Overall, our partnership with the local PHC and establishment of an on-site molecular laboratory enabled us to (I) conduct prospective longitudinal surveillance of the SAMIPS cohort and (II) link detailed clinical records with qPCR testing of nasopharyngeal (NP) samples. Critically, we designed SAMIPS to minimize selection biases by sampling on a fixed schedule rather than responding to apparent symptoms. This framework allowed us to assess the burden of symptomatic pertussis, as is generally done when only testing children who present with a clinical syndrome typical of pertussis, as well as asymptomatic infections. By sampling mothers and infants on the same schedule, we also gained insights into the epidemiology and clinical presentation of pertussis within mother/infant dyads.

Our previous work in this cohort has profiled a small number of the most sick infants (40), inspected the timeliness of DTP vaccine uptake in cohort infants (41), and described antibiotics administration across the cohort (42). We also quantified evidence for pertussis infection (EFI) across the full cohort, where we observed a strong correspondence of EFI between mothers and their infants (10). To our surprise, we also found a strong correspondence between EFI and mild symptoms (cough and/or coryza alone) in infants as well as mothers, suggesting the common occurrence of mild pertussis in infants. We were also intrigued by the detection of apparently asymptomatic pertussis among a subset of infants with strong EFI (10).

Our previous findings underscored the epidemiological importance of mild and asymptomatic pertussis, especially in low-resource settings, and highlighted the prospect of weaker IS481 qPCR signals as sensitive indicators of pertussis infections in both populations and individuals. Notably, we identified numerous individuals with strong evidence of pertussis infection within a single year in one community in Lusaka, despite the complete absence of any official pertussis case reports in all of Zambia since 2009 (43). In short, our results suggested a systematic inability to detect ongoing pertussis transmission at a regional and national level.

Our previous work, however, left several key questions unaddressed. Here we explore the detailed time-course of qPCR tests and respiratory symptoms within individual subjects and within mother/infant dyads. We also critically examine the fidelity of our qPCR-based pertussis testing in order to assess the reliability and robustness of our results. Finally, we profile a group of subjects with evidence of persistent colonization. Altogether, our results offer granular insights into otherwise invisible pertussis dynamics in this at-risk population of mothers and their infants.

Results

Study Overview

SAMIPS was designed to enroll, across one calendar year, all newborns and their mothers in Chawama compound, a low-resource peri-urban community with a population of approximately 150,000 people at the time of the study (2015). The study was conducted at the single no-cost public health clinic available to Chawama residents, where subjects were observed at enrollment and (ideally) at six additional scheduled study visits across infants' first 100 days of life (plus *ad hoc* sick visits). At each visit, we recorded vital signs, antibiotics prescriptions, the administration of routine vaccinations (including DTP doses 1-3), and self-reported and observed respiratory symptoms. Critically, we also collected nasopharyngeal (NP) samples at each visit that were retrospectively tested for pertussis (and other common respiratory pathogens) via qPCR at our lab located at Lusaka's University Teaching Hospital (UTH).

We lack maternal vaccination records, but note that WHO estimates indicate DTP3 coverage in excess of 80% back to 1985 (44). This fact, along with the potential of widespread under-reporting of transmission in pertussis cases in LMICs, leads us to believe that few (if any) mothers were immunologically naive at the beginning of the study. We also note that genetic sequencing of pertussis was not conducted due to its reliance (at the time) on culturing. For additional details, including cohort demographics, see Gill et al. (40), Gunning et al. (41) and Gill et al. (10).

Ultimately, SAMIPS enrolled nearly 2,000 mother/infant dyads and conducted qPCR tests on over 19,000 NP samples. We focus here on the subset of 1,315 dyads who attended at least 4 study visits, for a total of 17,408 unique NP samples across 8,704 dyad visits (including 410 unscheduled visits).

qPCR: detection versus signal strength

For clinical pertussis diagnosis, IS481 $Ct < 35$ is a frequently used threshold (15), yet experimental variation between individual tests, plates, and labs precludes a direct estimate of sample quantity from Ct value in the absence of rigorous controls (45, 46, 47). Accordingly, we use the detection and relative quantification of IS481 here to focus on disease surveillance and epidemiological inference. In this context, we first classify IS481 qPCR tests as either detections (i.e., $Ct < 45$) or non-detections (N.D.). As before (10), we further categorize detections by signal strength into three

arbitrary groups: weaker ($Ct \geq 43$), intermediate ($43 > Ct \geq 40$), and stronger ($40 > Ct$). *A priori*, we expect that stronger signals are more reliably detected and are more predictive of symptomatic illness, carriage, and disease transmission. We proceed with the assumption that all IS481 detections are *potentially* informative, and directly interrogate the information content of qPCR tests by their signal strength.

Overall, we observe detections in 9% of NP samples ($N=1,540$, Table 1bA). Most of these detections consist of weaker IS481 signals (median $Ct = 43.2$); $Ct < 40$ in 10.2% of detections ($N=159$), and $Ct < 35$ for 1.7% ($N=26$). In Figure 1A, we show the frequency of IS481 detections by Ct value for mothers vs infants. Notably, stronger IS481 signals ($Ct < 40$) are observed much more frequently in infants (10% of detections) vs mothers (3.7%). We also summarize the strongest observed IS481 signal by subject and dyad (Figure 1B), again highlighting a long tail of stronger IS481 signals in infants. In Figure 1C, we show the number of mothers, infants, and dyads with one or more detections across their course of study participation. In addition, Table 1B tabulates the frequency of co-detection within a dyad's visit ($OR=4.9$, 95%CI: 4.1-5.9). In all cases, the observed overlap between infants and their mothers is much higher than expected by random chance, illustrating how detections tend to cluster within dyads.

Study Timeline

We employed a rolling enrollment of the study cohort such that each dyad's participation spanned a portion of the full study period. Figure 2A illustrates the study participation of six example dyads, highlighting infrequent unscheduled visits along with the sometimes uneven timeline of scheduled visits relative to the intended schedule. This figure also highlights the potential clumping of detections over subjects' study participation, a point that we return to later. Figure 2B shows the number of NP samples (and associated qPCR tests) by calendar week and IS481 signal strength, highlighting the changing cohort size across the study period. Figure 2C shows the frequency of NP samples by IS481 signal strength from a generalized additive model (GAM) that accounts for the variable cohort size. As previously described (10), we observe a burst of stronger IS481 signals in June 2015 that is preceded by a more gradual surge of weaker signals in the previous six weeks.

Experimental re-testing

During the course of qPCR testing, an opportunistic subset of 1,468 NP samples was tested a second time, including 399 initial detections and 1,069 initial N.D.s. In this subset we observed a very low probability of follow-up detection given initial N.D. of 2.3%, suggesting a low false positive rate. By contrast, in samples where initial qPCR results (Ct_0) were stronger ($Ct_0 < 40$), we found a high probability of confirmation of 86%. In total, this subset revealed a relative risk of confirmation of 21.9 (95% CI: 14.7-32.6, see Table 2), indicating very high overall repeatability of IS481 detection.

These results prompted us to conduct a systematic evaluation of IS481 qPCR reliability as a function of signal strength. To do this, we re-tested a random subset of NP samples. We stratified by initial qPCR strength and across mothers and infants, drawing 10 NP samples per strata across 6 groups of Ct_0 (129 total retested NP samples). As expected, we found no differences between mothers and infants. In Figure 3A, we show the re-test confirmation frequency by Ct_0 strata: here, the proportion of confirmed detections rose rapidly from approximately 25% for $Ct_0 > 42$ to 58% for $41 > Ct_0 > 40$ and 89% for $40 > Ct_0$. We also illustrate the overall detection frequency for the full library of qPCR results (8.8%, dashed line), which we henceforth employ as a baseline "null frequency" of detection, representing a conservative upper bound on the qPCR false-positive rate.

In Figure 3B, we consider Ct_0 as a continuous predictor of qPCR re-test confirmation. We use a GAM to predict the odds of confirmation, and then normalize the resulting prediction by the null odds of detection (above) to obtain an estimated mean odds ratio of confirmation (ORoC). Notably, the ORoC's 95% CI ceases to overlap with 1 just below $Ct = 44$, showing that the odds of confirmation exceed the null odds. At $Ct_0 = 43$, the estimated ORoC increases to 3.1 (95% CI: 1.8-5.5)

Table 1.

a) Percent of samples by IS481 signal strength across 8,704 dyad visits. b) Odds ratio of co-detection (OR +95% CI; rows show mothers, columns show infants, N=8,704 unique dyad visits).

(a)					(b)			
	N.D.	[45,43]	(43,40]	(40,15]	N.D.	Detect	OR	
Mother	90.7	5.5	3.1	0.7	N.D.	7344	520	1.0
Infant	91.6	4.4	2.8	1.1	Detect	597	209	4.9 (4.1-5.9)

Figure 1. Overview of qPCR test results showing A) frequency of IS481 Ct values (by visit), B) frequency of peak IS481 values (by subject and dyad), and C) number of IS481 detections across full study (by dyad).

A) Intermediate and stronger IS481 signals (color) were much more common in infants than mothers (inset numbers show cumulative frequency from left to right; Ct < 35 not shown). B) More infants exhibit stronger peak IS481 values, while dyad members together frequently exhibit weaker peak signals (dashed line). C) Venn diagrams show the frequency of subjects with ≥ 1 (left) to 4 (right) detections (Ct < 45), stratified by infants, mothers, and both dyad members (shaded region).

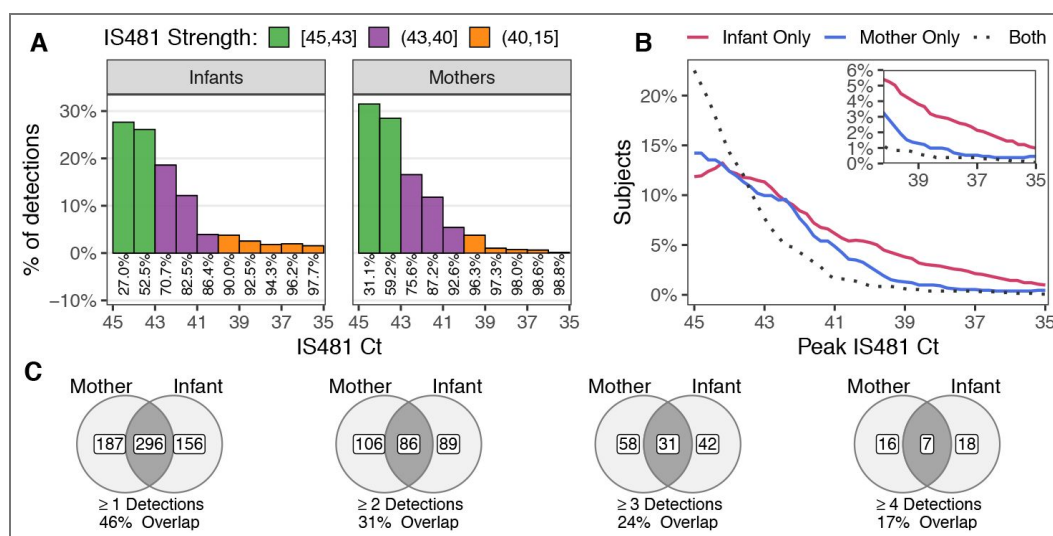


Figure 2. Study timeline.

A) Timelines of select dyads (mother/infant pairs) showing IS481 qPCR signal strength. Dyad study visits were scheduled at approximately two week intervals (median 16 days). B) Subject visits per week, stratified by IS481 signal strength (color, grey shows N.D.). C) Estimated frequency of IS481 detections by signal strength over time (GAM, mean +95% CI).

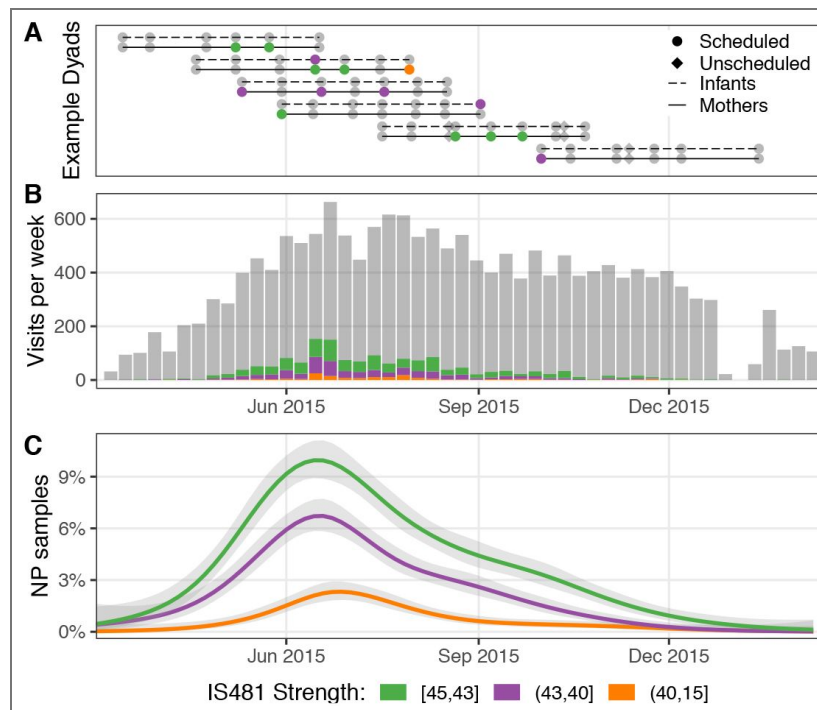
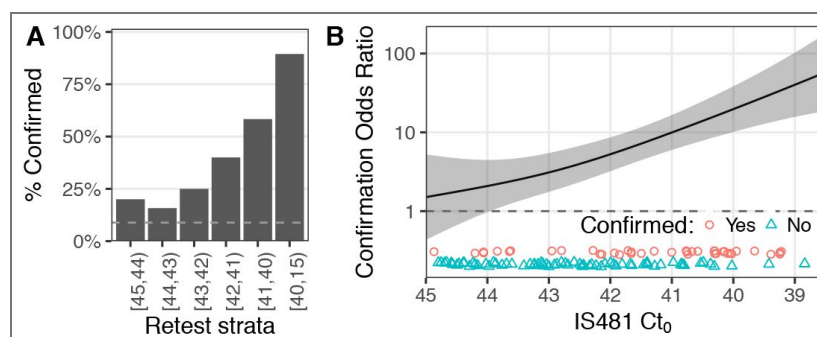


Table 2. Relative risk of confirmation (columns) given initial detection (rows) in an opportunistic subset of 1,468 NP samples that were retested.

	N.D.	Detect	RR
N.D.	1044	25	1.0
Detect	195	204	21.9 (14.7-32.6)

Figure 3. In a stratified random subsample of 122 NP samples with initial detections, re-test confirmation frequency increased with initial IS481 signal strength.

A) shows confirmation frequency by subsample strata. B) Odds ratio of confirmation by IS481 signal strength (OR relative to null frequency of detection across full sample library; mean and 95% CI estimated from a binomial GLM). Points show initial IS481 Ct stratified by confirmation (shape, color).



and at $Ct_0 = 40$ to 19.7 (95% CI: 10.1-38.3). In summary, confirmation was more likely than expected by chance alone (i.e., than the null frequency of detection observed in the full NP sample library) across the full spectrum of Ct values, including even weaker signals.

We note that the above results are consistent with the expected stochastic variation intrinsic to qPCR. For example, assuming a realistic PCR efficiency of 1.7, we could expect a Ct of 42 given 10 targets per sample (i.e., at the theoretical limit of quantification; see Fig. 5 in Ruiz-Villalba et al. (47)). We would also expect Poisson sampling variation in perreaction target number to yield an approximately continuous decrease in re-test confirmation probability with increasing Ct , as observed here (e.g., see Fig. 9 in Ruiz-Villalba et al. (47)).

Quantifying the information content of IS481 signal strength

The preceding results bolster our confidence that all qPCR tests are *potentially* informative, particularly for epidemiological and public health surveillance where sensitivity often takes precedence over specificity. With this in mind, we next assess the information content of qPCR tests within the longitudinal context of both individuals and mother/infant dyads.

Time to first detection: reciprocal impacts within dyads

We first employ a survival analysis framework to investigate how the time to initial detection in one dyad member (the *ego*) is affected by preceding IS481 signals within their *alter* (i.e., the ego and alter represent *either* mother and infant, or infant and mother). Here, model covariates include the ego's identity (infant versus mother) and the alter's IS481 signal strength at the preceding visit (N.D., $Ct \geq 43$, $43 > Ct \geq 40$, and $40 > Ct$). Along with the first detection of *any* IS481 signal (model A: $C \leq 45$), we also construct survival models predicting the time to first occurrence of intermediate and stronger IS481 signals (model B: $Ct \leq 43$ and model C: $Ct \leq 40$, respectively).

As we move from model A to model C, we focus on greater outcome specificity at the expense of fewer events and lower sensitivity. Overall, we find stronger evidence that infants (ego) are affected by mothers (alter) than vice versa. Figure 4 shows the hazard ratio (HR, Y axis) of each event (color) conditioned on the alter's preceding visit (X axis), where the HR is relative to an N.D. in the alter's preceding visit. Notably, we find that weaker IS481 signals in mothers' preceding visit still predict elevated HRs in infants: $Ct \leq 45$, HR=1.9 (1.3-2.8 95%CI); $Ct \leq 43$, HR=2.0 (1.2-3.3 95%CI); $Ct \leq 40$, HR=2.5 (0.98-6.1 95%CI). And, despite wide confidence intervals from infrequent events, a greatly increased HR of $Ct \leq 40$ in both infants and mothers is predicted from preceding intermediate IS481 signals in their alters: infants, HR=6.3 (2.6-14.7 95%CI); mothers, HR=5.4 (1.9-14.2 95%CI).

These results are broadly consistent with the assumption that mothers more frequently transmit pertussis to their infants than vice versa. Somewhat surprisingly, even weak signals in mothers correspond to elevated HRs in infants. And, while our study design can't exclude the possibility of a shared source of infection, the time-ordered nature of these results offers a potentially useful clinical indicator for infant risk.

Statistical associations within subjects and dyads

While the above survival models suggest a causal role of mothers' IS481 signals on infants and (to a lesser extent) vice versa, these models only consider the first occurrence of each event. We next sought to characterize the full time course of each subject by considering two binary experimental outcomes: 1) future detections **within** subjects, and 2) contemporaneous detections within each dyad visit. We report the relative risk (RR) of each outcome stratified by IS481 signal strength, using N.D. as the baseline group.

For outcome 1, we predict future detections from each subject's *own* IS481 signals at the preceding visit. For infants (Table 3a), we find an increase in the RR of detection from 2.3 (95% CI: 1.9-3.1) for weaker IS481 signals ($Ct \geq 43$) to 4.9 (95% CI: 3.7-6.5) for stronger signals ($Ct < 40$), and very little difference between infants and mothers (Table 3b).

Figure 4. Survival analysis, showing hazard ratios (HR) of each event (color) in an ego conditioned on the alter's IS481 signal in the previous visit (X axis; HR relative to N.D.

in alter). Overall, HRs are higher in infants than mothers and are significantly greater than 1 in most cases (asterisk indicates 95% CI non-overlap with 1). Here 'event' denotes the first occurrence of an IS481 signal at least as strong as indicated. Infrequent events for the strongest signals yield large CI ($Ct \leq 40$, upper limit not shown).

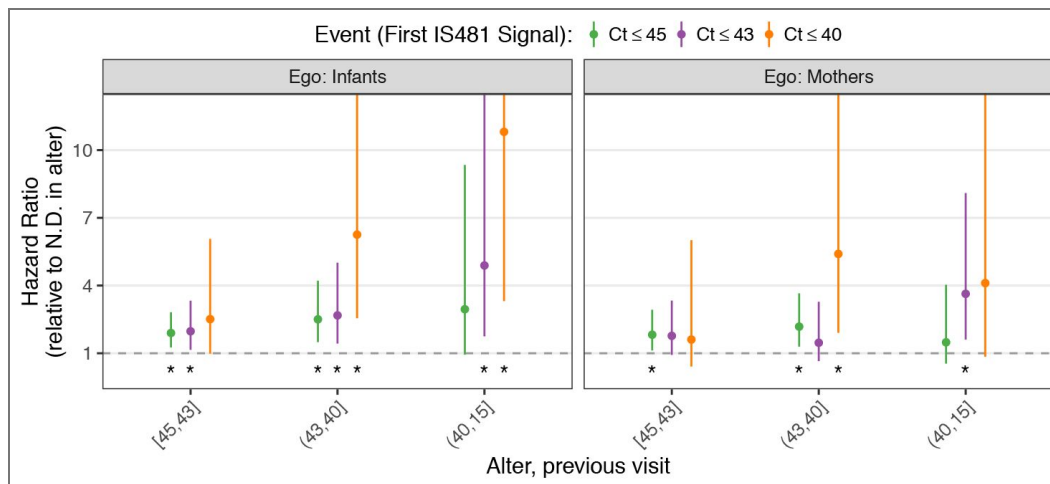


Table 3. Relative risk of detection (RR +95%CI) per visit is greater given stronger IS481 Ct values in previous visits (no significant difference between (a) mothers and (b) infants).

(a)				(b)			
Infant IS481	N.D.	Detect	RR	Mother IS481	N.D.	Detect	RR
N.D.	6240	525	1.0	N.D.	6159	527	1.0
[45,43]	263	62	2.5 (1.9-3.1)	[45,43]	338	76	2.3 (1.9-2.9)
(43,40]	159	55	3.3 (2.6-4.2)	(43,40]	174	62	3.3 (2.7-4.2)
(40,15]	53	33	4.9 (3.7-6.5)	(40,15]	33	19	4.6 (3.2-6.7)

For outcome 2, we predict detections in the ego from contemporaneous IS481 signals in their alter. We find a similar pattern as in outcome 1, where IS481 signals of mothers (alter) predict detections in infants (ego) and vice versa. Yet here we find notable differences between infants and mothers. For infants (Table 4a), we again find the RR of detection increasing from 2.7 (95% CI: 2.2-3.3) for weaker alter IS481 signals ($Ct \geq 43$) to 8.1 (95% CI: 6.3-10.4) for stronger signals ($Ct < 40$). We observe a more modest impact of infant Ct values on detections in mothers (Table 4b).

Respiratory symptoms

Our final analysis of the full cohort investigates IS481 signal strength as a predictor of attendant respiratory symptoms. As above, we report RR for mothers and infants separately for *any* respiratory symptoms (predominantly cough and/or coryza). For infants, we also employ a set of GAMs to estimate the frequency of symptoms as a (continuous) response to IS481 signal strength. Here we focus on visits with IS481 detections (i.e., excluding N.D.s) and construct separate models for a) *any* respiratory symptoms and b) serious symptoms (i.e., excluding cough and/or coryza).

Unsurprisingly, we recorded respiratory symptoms far more frequently in infants than in mothers, regardless of IS481 signal strength (Table 5). Overall, we find that IS481 signals strongly predict symptoms in infants but only weakly in mothers. For infants (Table 5a), we see an increase in the RR of symptoms from 1.5 (95% CI: 1.2-1.9) for weaker IS481 signals ($Ct \geq 43$) to 5.1 (95% CI: 4.3-6.1) for stronger signals ($40 > Ct$). For mothers, we observe a (marginally significant) RR of symptoms that increases from 1.6 (95% CI: 1.1-2.3) for weaker IS481 signals to 2.4 (95% CI: 1.1-5.1) for stronger signals.

Focusing on infant visits with detections, Figure 5 shows the quantitative response of symptom frequency to IS481 Ct . The frequency of *any* symptom increases from 13% (95% CI: 9-18%) to 46% (95% CI: 40-52%) for Ct values of 45 and 40, respectively. While serious symptoms are much less common overall, again we find an increase from 2.5% (95% CI: 1.3-4.7%, $Ct = 45$) to 9% (95% CI: 6-13%, $Ct = 40$). We note that comparable models for mothers show no statistically significant association between IS481 Ct and frequency of respiratory symptoms.

Taken together, these results quantify how IS481 signals cluster in time, within dyad visits, and correspond with respiratory symptoms. Notably, even the weakest IS481 signals ($Ct \geq 43$) are associated with statistically significant increases in the above outcomes (i.e., RR relative to N.D., Tables 3-5). We also observe noteworthy differences between infants and mothers, particularly for the time to first detection (Figure 4) and respiratory symptoms (Table 5 and Figure 5).

Persistent colonization: evidence from a subgroup of subjects

The above analyses suggest an important role of false negatives in interpreting the time-course of individual subjects. Yet this potential ambiguity of qPCR signals complicates subject-level interpretation of infection status over time. Throughout the preceding analyses we have examined the full cohort, where IS481 signals are predominantly mild and infrequent (e.g., Fig 1C). To conclude, we examine those subjects with strong and/or frequent qPCR signals. In this way we focus on the time-course of subjects with *strong evidence of infection* (10), thereby reducing the relative impact of false negative qPCR tests.

We begin with a detailed view of 20 subjects (10 infants and 10 mothers) with the strongest *peak* IS481 signals, shown in Figure 6 (subjects ordered by number of detections). Clustering of detections around strong IS481 signals ($Ct < 35$) is evident here, consistent with our previous findings in this cohort of stepwise transitions of IS481 signals within subjects (i.e., Figure 6 in Gill and Gunning et al. (10)). However, uninterrupted runs of detections are uncommon. We also note that respiratory symptoms are common but not consistently observed in this group of subjects.

With the above in mind, we sought to identify subjects with evidence of *persistent colonization*, which we define here as a cluster of ≥ 3 detections that lacks two or more adjacent N.D. (that is, we permit isolated but not consecutive N.D.s). In this way, we seek to balance sensitivity (including

Table 4.

Relative risk of contemporaneous detection (RR +95%CI) in ego given IS481 signal strength in alter: a) infant risk given mother's IS481; b) mother's risk given infant's IS481. Overall, stronger signals predict more frequent detections, with modest differences between the predictive strength of IS481 signals in mothers a) versus infants b).

(a)				(b)			
Mother IS481	N.D.	Detect	RR	Infant IS481	N.D.	Detect	RR
N.D.	7344	520	1.0	N.D.	7344	597	1.0
[45,43]	392	85	2.7 (2.2-3.3)	[45,43]	293	91	3.2 (2.6-3.8)
(43,40]	177	92	5.2 (4.3-6.2)	(43,40]	166	79	4.3 (3.5-5.2)
(40,15]	28	32	8.1 (6.3-10.4)	(40,15]	61	39	5.2 (4.0-6.7)

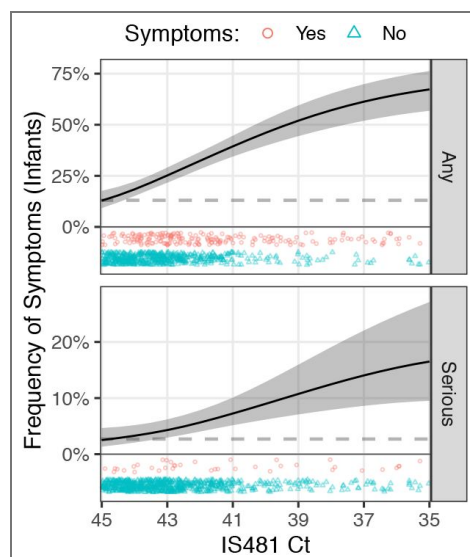
Table 5. Relative risk of respiratory symptoms by IS481 signal strength for a) infants and b) mothers.

No symptoms: N.S.

(a)				(b)			
	N.S.	Symp	RR		N.S.	Symp	RR
N.D.	7049	923	1.0	N.D.	6412	304	1.0
[45,43]	317	68	1.5 (1.2-1.9)	[45,43]	382	29	1.6 (1.1-2.3)
(43,40]	164	84	2.9 (2.4-3.5)	(43,40]	202	16	1.6 (1.0-2.6)
(40,15]	41	59	5.1 (4.3-6.1)	(40,15]	50	6	2.4 (1.1-5.1)

Figure 5. The frequency of reported respiratory symptoms increases with stronger IS481 signals among infant visits with IS481 detections (N=733), both for any symptoms (top) and serious symptoms (bottom: excludes solely cough and/or coryza).

Curves show mean and 95% CI estimated from GAMs (one per row). Dashed lines shows marginal frequency of symptoms in infants (Any=13%; Serious=2.7%; N=8,705). Points show presence of respective symptoms by Ct value.



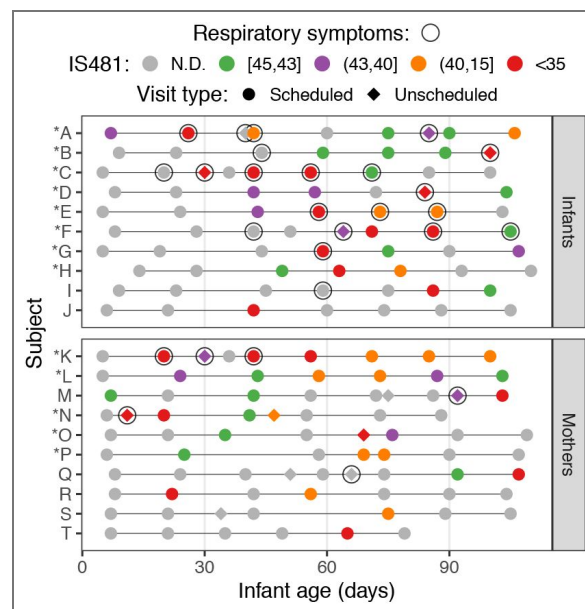


Figure 6. Timelines of study visits showing IS481 signals (color) for the ten infants (A-J) and ten mothers (K-T) with the strongest peak IS481 signals within each group.

Peak IS481 Ct in these infants ranges from 18.1 to 32.6 (mean=27.1) and from 30.0 to 36.4 in these mothers (mean=33.7). Clusters of detections are evident that, however, frequently contain N.D.s. Within each panel, subjects are ordered by detection frequency. Open circles mark visits with any reported respiratory symptoms. Asterisks indicate inclusion in the subgroup analysis below (Fig 7), including 8 of 10 infants and 5 of 10 mothers.

subjects with persistent evidence of IS481) with specificity (focusing on subjects where detections are clustered in time). Using these criteria, we identified a subgroup of 50 infants and 54 mothers. Despite not accounting for IS481 signal strength, this subgroup includes 8 infants and 5 mothers from [Figure 6](#) (marked with asterisks above), showing that our duration-based criteria also capture many subjects of those exhibiting the strongest IS481 signals.

We provide a detailed analysis of this subgroup in [Figure 7](#). [Figure 7A](#) displays the Kaplan-Meier estimated duration of detection, where no difference between infants and mothers is evident (median duration of 55 days, 95% CI: 46-69 (infants) and 50-68 days (mothers)). [Figure 7B](#) provides a detailed view of censoring, which was especially common in these mothers (where both left and right censoring occurred). Notably, the precision of the above duration estimates are limited by frequent right censoring (i.e., detection at last visit), which was observed in 20 infants and 20 mothers in the subgroup. Regardless, we find substantial evidence of persistent colonization, including 5 infants and 7 mothers with detections lasting ≥ 10 weeks (with right censoring in 5 of each).

In [Figures 7C-D](#), we illustrate peak IS481 *Ct* and frequency of respiratory symptoms. [Figure 7C](#) shows that peak IS481 *Ct* values are *much* higher in this subgroup than the full cohort (i.e., [Figure 1B](#)), and higher here in infants than mothers. [Figure 7D](#) highlights the relative sparsity of respiratory symptoms in this subgroup. We were particularly surprised to find that 22% of infants ($N=11$) and 65% of mothers ($N=35$) reported no symptoms across their study participation. In addition, of the 28 infants and 16 mothers with strong IS481 signals ($Ct < 40$, [Figure 7C](#)), only 9 infants (32%) and 1 mother (6%) reported *any* serious respiratory symptoms.

Overall, we find strong evidence of persistent colonization in this subgroup, including strong peak IS481 signals and long durations of detection. In addition, frequent censoring shows that our 100-day follow-up period was not long enough to fully characterize the *maximum* duration of colonization, as evidenced by [Figure 7A](#) and [7C](#). Yet we observe minimal respiratory symptoms among this subgroup, even among infants. In addition, we find that a majority of these mothers reported no symptoms at all.

Discussion

Pertussis remains a leading vaccine-preventable disease that is endemic worldwide, and whose global impacts have been underscored by its dramatic post-pandemic resurgence across a range of countries ([48](#), [49](#), [50](#), [51](#), [1](#)). This is despite generally high national vaccine coverage ([44](#), [1](#)), though considerable variation in vaccine uptake and timing exists worldwide ([52](#), [53](#), [54](#), [41](#), [1](#)). Further complicating this picture are major gaps in pertussis surveillance at the national and sub-national level, particularly in LMICs, as well as rapidly shifting diagnostic methods and standards ([36](#), [55](#), [56](#), [57](#)).

Meanwhile, mounting evidence of vaccine-driven evolution in *B. pertussis*, along with widespread antibiotic resistance in some regions, calls into question whether current clinical and public health tools will remain effective in the near future ([58](#), [59](#), [26](#)).

Within this milieu, sub-Saharan Africa represents a particular challenge, where poorly-resourced primary health care intersects with minimal surveillance and high disease burdens. For example, recent work revealed an almost complete lack of genomic surveillance within Africa, limiting conclusions about important aspects of pertussis epidemiology such as evolution of vaccine or antibiotics resistance ([26](#), [57](#)). Meanwhile, high fertility rates and HIV positivity throughout the region add to the supply of human hosts for disease transmission ([60](#), [61](#), [62](#)).

To successfully address the many public health challenges posed by pertussis, our results indicate that a clearer view of national and sub-national pertussis dynamics is needed, especially in low-resource settings where the burden of disease is highest. For example, our work points to the possibility of replacing symptoms-based pertussis surveillance with population-level qPCR-based prospective surveillance, which could then inform clinical care. In these regards, Lusaka is

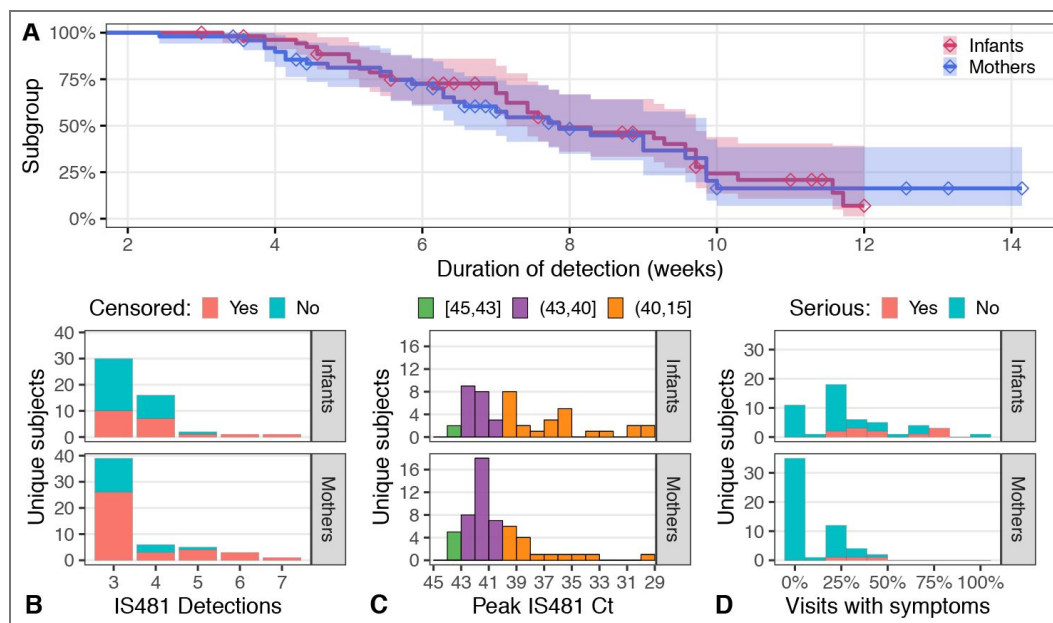


Figure 7. Subgroup analysis: subjects with evidence of persistent colonization, including 50 infants and 54 mothers.

A) Kaplan-Meier survival analysis of duration of detection (i.e., time interval between the first and last detection) that accounts for right censoring (detection at final study visit, shown by diamonds; includes 20 infants and 20 mothers). Estimated median duration of detection was 55 days. Panels B-D show the number of subjects by (B) number of detections, (C) peak IS481 signal, and (D) frequency of visits with respiratory symptoms. B) Number of detections stratified by censoring (color). For mothers, both left and right censoring are included (i.e., detection at first and last visit). C) Peak IS481 Ct is much higher in infants than mothers and, unlike the full cohort (Figure 1B), is rarely a weaker signal (green: Ct >43). Not shown: 3 infants with peak Ct ranging from 23.4 to 18.1. D) Most subgroup infants experienced respiratory symptoms infrequently, while most subgroup mothers experienced no respiratory symptoms. Color shows serious symptoms (i.e., excluding simple cough and/or coryza), which occurred most frequently in a small number of infants.

broadly representative of the urbanized Global South, where routine childhood vaccination coverage is generally high but community surveillance and clinical testing for pertussis is largely absent and prenatal maternal vaccination is costprohibitive (63).

In our cohort of mothers and their newborn infants, we rarely observed severe disease (see also Gill et al. (40)) but were surprised by the high overall frequency of IS481 detections, including a substantial number of intermediate and stronger IS481 signals (Figure 1). We commonly observed contemporaneous detections within mother-infant pairs (Table 1b), and we documented more frequent respiratory symptoms in those infants (but not mothers) with stronger IS481 signals (Table 5, Figure 5). We could not confidently attribute directionality of infection within dyads, nor can we definitively rule out shared environmental exposure. However, our survival analysis points to a greater impact of mothers on their infants than vice versa (Figure 4), a directionality that suggests causality.

Finally, we identified a subgroup of some 50 mothers and infants who displayed evidence of persistent colonization (median duration of approximately eight weeks), in which most mothers were entirely asymptomatic (Figure 7D). Taken together, our results support a picture of episodic but widespread pertussis transmission within the broader Chawama community that includes transmission from mostly asymptomatic mothers to their young infants.

Throughout this work, we have focused on mild and minimally symptomatic pertussis, as classical pertussis was rare in our cohort (40). As noted above, we do not directly distinguish carriage from transmissible infection here, nor do we establish direct epidemiological linkage between cases. However, we can confidently state that A) evidence of infection (here, stronger IS481 signals) was much less common in mothers than in infants (Figure 1A-B) and that B) infants who experienced these stronger maternal IS481 signals were at greater risk of later exhibiting evidence of infection themselves (Figure 4). Furthermore, while both mild and more serious respiratory symptoms were commonly observed in infants with stronger IS481 signals, this pattern was largely absent for mothers (Table 5b and Figure 5 and 7). Overall, our results suggest that transmission from mothers to infants remains uncommon but that such transmission can nonetheless occur in the absence of symptomatic infection.

Test reliability, disease surveillance, and public health

In this study, we exclusively employed qPCR-based testing due to its high sensitivity, relatively low cost, and minimal burden on study participants. In order to better interpret individual test results, we leveraged the temporal context of our longitudinal sampling scheme. With this approach, we considered all qPCR detections as potentially informative, allowing us to directly interrogate the predictive power of weaker versus stronger qPCR signals. As such, we do not directly distinguish carriage from transmissible infection (apart from statistical evidence, see Figure 4). We note, however, that the detailed relationship between exposure, carriage, and transmissible infection remains poorly characterized even when serological surveys are available (38, 39).

Considerable debate exists within the pertussis literature about the use of specific C_t thresholds for interpreting IS481 qPCR results in both clinical and epidemiological contexts (64, 65, 66, 15, 56). Our retesting results (Table 2 and Figure 3) emphasize the overall high fidelity of the IS481 qPCR assays conducted in our lab while also highlighting the sampling variation intrinsic in this assay (67, 65). In modern laboratory settings, this uncertainty is commonly addressed via repeat testing and qPCR efficiency correction (45, 47, 46). However, disease surveillance efforts are often time- and resource-constrained, especially in low-resource settings such as Lusaka. With this in mind, our central goal was *not* to directly quantify IS481 copy number, but rather to reliably identify and characterize pertussis infections in subjects. In this context, our limited qPCR retesting provides a benchmark for repeatability in this system and points to an important C_t threshold ($C_t = 40$) below which stochastic non-detections were rare in our lab.

In assessing the information content of weaker qPCR signals, some caveats are warranted. First and foremost, caution should be exercised in comparing C_t values between labs in the absence of objective standards and/or qPCR efficiency corrections (46, 68). We suspect that our average qPCR

efficiency was lower than that of better-resourced laboratories, which would yield larger Ct values for a given sample. Notably, lower qPCR efficiency could partly explain the abundance of very weak IS481 signals in our results. Unfortunately, we did not retain PCR efficiency information as part of our laboratory pipeline. We recommend that future efforts prioritize reporting this information in accordance with modern best practices for Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (45).

We also note that interpretation of weak IS481 qPCR signals must account for the high copy number of this repetitive element. For example, our laboratory protocols specified that 1/50 of the DNA extracted from an NP sample was included in each qPCR test. Given a conservative estimate of 240 IS481 copies per genome, an NP sample that captured 10 bacteria would yield approximately 48 copies per qPCR test, well above the so-called qPCR limit of quantification of ten targets per reaction (see Fig. 8 in Ruiz-Villalba et al. (47)).

The above estimates stand in sharp contrast to the widely-cited claims of Tatti et al. (69), who write: “On the basis of our analytical sensitivity data ... IS481 CT values in the range of $35 < CT < 40$ indicate the presence of less than 1 bacterium per reaction, which we consider uninterpretable.” As we have detailed above, Ct values that represent fewer than one genome equivalent contain valuable information and should be expected in carefully-conducted *B. pertussis* surveillance efforts. We recognize that such weak and potentially ambiguous signals may not be appropriate for clinical diagnosis. However, our results demonstrate that they nonetheless contain valuable information about pathogen presence and infection intensity that can (and should) be leveraged for disease surveillance.

Assessing population immunity (and susceptibility)

An important outstanding question from our study is the role of repeated pertussis infections in immune boosting. If we assume that 2015 was a typical year in Lusaka and that every mother who experienced ≥ 3 detections experienced a clinical infection (Figure 1C), then we estimate an annual attack rate of 6.8% of mothers. This corresponds to a mean return frequency of one infection per 14.8 years, which is much shorter than the presumed duration of immunity from natural infection or the whole-cell pertussis vaccination used in Zambia (70, 71). We note, however, ongoing debate surrounding the relative quality (and duration) of protection provided by natural infection, whole-cell vaccination and, more recently, a range of acellular vaccine formulations (72, 73, 74, 4, 1). We also note that pregnancy impacts immune functioning (75), such that the postpartum mothers in our cohort are not necessarily representative of the broader Chawama population. Nonetheless, our results point to episodically high community prevalence that could, in turn, drive immune boosting this population.

Unfortunately, the absence of unambiguous serological correlates of protection makes the confident assessment of population immunity via serosurveys challenging in the best of circumstances (76, 77, 39). And, while serological surveillance has been successfully deployed in similar settings (37), in our own field experiences, we found it much easier to increase the cohort size and frequency of clinic visits in the absence of blood draws and processing. With this in mind, we suggest that qPCR-based prospective disease surveillance offers a powerful and cost-efficient tool, both for ongoing pertussis surveillance and intervention activities as well as long-term research into pertussis transmission dynamics.

Implications for global public health

Pertussis vaccine uptake remains a key country-level metric in tracking infectious disease prevention worldwide, led by the WHO and GAVI (78, 54, 79). However, much less is known about the true burden of pertussis infections in LMICs (63, 80, 81). In this context, pertussis surveillance in Lusaka is broadly representative of urban low-resource settings worldwide, where active surveillance is largely absent and passive surveillance only captures the most severe symptomatic illness. Indeed, the majority of active pertussis surveillance (and research) at present is focused on high-income countries where incidence is least common. In order to fully understand the

“pertussis enigma” of sustained disease transmission in the face of high levels of presumed immunity from infection and/or vaccination, we expect that researchers will need to alter existing strategies and broaden their horizons.

As a populous, middle-income and primarily urban country, Zambia offers an evocative example of pertussis surveillance, where no cases have appeared in official WHO reports since 2009 (43). Yet by following a modest-sized cohort of 1,315 mothers and their newborn infants across a single year, we identified 55 infants (4.2%) and 50 mothers (3.8%) with evidence of persistent colonization, most of whom were minimally symptomatic. Taken together, these findings reaffirm the overall success of Zambia’s whole-cell pertussis immunization schedule in protecting against symptomatic disease. Moreover, our results are consistent with broad-based reductions in pertussis transmission at the population level. Yet these findings underscore both the stubborn persistence of pertussis within Zambia along with shortcomings of symptoms-based pertussis surveillance.

Our findings in Lusaka’s Chawama compound broadly align with the few available African studies of pertussis, including Uganda (15-20% prevalence in children with persistent coughs)(82) and South Africa’s notable PHIRST study (5.8-9.6% prevalence, mostly asymptomatic, in a prospective longitudinal household cohort) (56, 37) (see also Soofie et al. (83)). Overall, our results point to a long-overlooked “invisible mass” of minimally symptomatic pertussis incidence below the visible iceberg of officially recorded pertussis cases (84).

We believe our study also offers a template whereby high-quality disease surveillance can be achieved through efficient use of in-country public health resources. At various points we faced challenges in local capacity building and the timely processing of samples. Nonetheless, we believe that our strategy could be implemented at scale in a cost- and resource-efficient manner in LMICs, for example, through coordination of public health clinics with a central diagnostic laboratory, as we did. Such an active surveillance program would shed invaluable light on the total burden of pertussis incidence in low-resource settings, inform clinical treatment, and ultimately illuminate the factors that drive sustained transmission. Such a program could also complement local public health efforts, for example, by calibrating ongoing pertussis intervention efforts, and could yield further dividends by increasing public trust in government and building local biomedical diagnostic capacity (85).

Materials and Methods

Study Design

We provide a detailed account of study methods, including sample size considerations and inclusion criteria, in Gill et al. (40) and Gill et al. (10). We offer a brief overview here.

SAMIPS was designed as a longitudinal cohort study that followed mother/infant dyads across infants’ first three months of life. The study was based in Chawama compound, a densely populated low-resource peri-urban community near central Lusaka, Zambia. SAMIPS sought to enroll all healthy live births that occurred between March and December 2015 in Chawama, and included a public outreach campaign towards pregnant Chawama residents. Subjects were enrolled at the Chawama Primary Health Clinic (PHC), which is the only government-supported clinic in this community and primary source of medical care for Chawama residents.

Mother/infant dyads were recruited at their first scheduled postpartum well-child visit (approximately 1 week of age). SAMIPS incentivized mothers to join and remain in the cohort by A) provided concierge (no-wait) routine and acute medical care for participants while enrolled, B) providing mothers with a per-visit travel stipend valued at approximately 7 US dollars and C), providing a small gift of baby supplies at the final scheduled study visit.

Each subject was scheduled to attend seven routine study visits, included a baseline enrollment visit and six follow-up visits spaced at 2–3 week intervals through 14 weeks old (maximum, 18 weeks). Additional unscheduled clinic visits could be initiated by mothers for acute medical care and/or routine well-child care. At each visit, we A) obtained nasopharyngeal (NP) swab samples

from both mother and infant, B) recorded self-reported respiratory symptoms a standardized reporting sheet, and C) record administration of infant vaccinations and antibiotics (if any). To avoid possible contamination, routine childhood vaccinations were provided in a separate area of the clinic compound after NP sampling was conducted. We used Unique barcodes from pre-printed sticker books to track study records, thereby linking subjects, clinic visit records, and NP samples. qPCR results were unavailable during the study and thus could not affect clinical treatment.

The SAMIPS study received ethical approval from the Boston University School of Medicine IRB and the ERES Converge IRB in Lusaka. The data sets used in this analysis went through a de-identification process at the request of NIH, and no personally identifiable information is used here.

Sample Collection and Processing

We collected NP samples using flocked-tipped nylon swabs (Copan Diagnostics, Merrieta, California). NP samples were collected from the PHC clinic daily and transported to our PCR laboratory at the University Teaching Hospital (UTH) and were stored at -80°C . DNA was extracted using the NucliSENS EasyMag system (bioMérieux, Marcy l'Etoile, France). Extracted DNA from each NP sample was tested for the IS481 insertion sequence and the constitutively expressed human RNase P (RNP) to assess sample collection, storage, DNA extraction, and qPCR reaction. Each 96-well qPCR plate contained a negative and IS481-positive control, along with approximately 46 samples (one each of IS481 and RNP). Reactions were run for 45 cycles on either a ABI 7500 thermocycler (ThermoFisher Scientific Inc, Waltham, MA) or a QuantStudio5 thermocycler (ThermoFisher Scientific Inc, Waltham, MA). We inspected samples run in parallel on both machines and found minimal systematic variation between machines. All primers and probes were purchased from Life Sciences Solutions (a subsidiary of ThermoFisher Scientific Inc). For sample retests, a random subset of NP samples was drawn from the analysis set sample library stratified by IS481: we selected NP samples from 10 mothers and 10 infants across 5 evenly-spaced IS481 Ct intervals from $Ct = 45$ to $Ct = 40$, plus an additional interval of $40 > Ct$.

Statistical Analysis

Similar to our previous work (10), we focus here on dyads where both subjects had ≥ 4 fully-documented study visits. We exclude 6 mothers and 6 infants from our previous analysis set (i.e., Table 1 in Gill et al. (10)) where sufficient records for the full dyad was not available. All analysis was conducted in R 4.2.2 (86).

As in previous work, we define IS481 *detection* as any detectable Ct value ($Ct > 45$). We further categorized IS481 Ct values into *IS481 signal strength*: “no detection” (N.D.), weaker ($Ct > 43$), intermediate ($43 > Ct > 40$) and stronger ($40 > Ct$). These divisions were based on manual inspection of the empirical distribution of Ct values (Figure 1A) and model results from retest data (Figure 3B). Of note, we employ categorical IS481 signal strengths in order to compute relative risks for various quantities using N.D. as the reference level.

We also construct a set of linear models that employ IS481 Ct values as continuous predictors. We exclude N.D.s from these models, i.e., conditioning on detection. Generalized additive models (GAM) were constructed using the mgcv package (87). Confidence intervals for all linear models were computed via estimated marginal means using the emmeans package (88).

We constructed a three separate survival models for our (within subject) time-to-event analyses using a Weibull distribution. For each signal strength category, we predict the time (in days) to the first detection of at least that signal strength. Predictors include whether the subject is an infant or a mother and the alter’s categorical IS481 signal strength at the previous visit (and their interaction). We also tested models that included alters’ respiratory symptoms. Survival models and hazard ratios were fit using the flexsurv package (89). Hazard ratio confidence intervals were constructed via bootstrap simulations using 10,000 samples.

Data availability

All data and code are available at <https://osf.io/zjy4t> .

Data Availability

Data available at <https://osf.io/wya75/> .

<https://osf.io/zjy4t> .

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Peer reviews

Reviewer #1 (Public review):

Summary:

The study investigates the role of asymptomatic pertussis carriage in transmission between mothers and their infants in particular. The authors use a longitudinal cohort study that involved 1,315 mother-infant dyads in Lusaka Zambia and they utilized qPCR based detection of IS481 to track Bordetella pertussis transmission over time. Insights from the study suggest that minimally symptomatic or asymptomatic mothers may act as a reservoir for B. pertussis transmission in the infants thus challenging the traditional surveillance methods that focus on symptomatic cases. Additionally, the study also identified a subgroup of persistently colonized individuals where mothers were majorly asymptomatic despite sustained bacterial presence.

The authors aimed to improve comprehension of pertussis transmission dynamics in high burden low resource settings and they advocated for an enhanced molecular surveillance strategies to capture full pertussis infection including those that might have gone undetected.

Strengths:

The strength are the use of innovative study design especially the longitudinal approach and routine sampling rather than symptom driven testing that minimizes bias in the study. The methodology were also rigorous and transparent by evaluating IS481 signal strength to classify pertussis detection and conducts retesting to assess qPCR reliability. There was also important epidemiological insights and the findings challenge the traditional wisdom by suggesting that pertussis transmission may frequently occur outside of symptomatic cases. The findings also showed its relevance to global health and policy by arguing for the incorporation of molecular tools like qPCR for surveillance of pertussis in low resource setting.

Weaknesses:

These includes reliability on qPCR based detection without additional validation measures like confirmatory culture or serology. There are also potential alternate explanation for transmission patterns observed in the study such as shared environmental exposure or household transmission. Additionally, there are limited generalizability as the study was done in a single urban site in Zambia. There is also lack of functional immune data.

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Reviewer #2 (Public review):

Summary:

In this paper, the authors describe the results of a longitudinal study of pertussis infection in mother/infant dyads in Lusaka, Zambia. Unlike many past studies, the authors assessed the infection status of individuals independently of whether they were symptomatic for a respiratory infection. As a result, this work represents one of the first studies specifically designed to assess asymptomatic transmission of pertussis. Using qPCR, the authors find strong evidence for the role of asymptomatic transmission from mothers to infants and also evidence for long-term bacterial carriage. This work represents an important contribution to our understanding of the global burden of pertussis. Also, it highlights the still under-appreciated role of asymptomatic transmission across many infectious diseases (including vaccine-preventable ones).

Strengths:

Unlike many past studies, the authors assessed the infection status of individuals independently of whether they were symptomatic for a respiratory infection. As a result, this work represents one of the first studies specifically designed to assess asymptomatic transmission of pertussis. Using qPCR, the authors find strong evidence for the role of asymptomatic transmission from mothers to infants and also evidence for long-term bacterial carriage.

Comments on revised version:

I appreciate the authors' attention to my comments during the revision process and still believe that their work represents an important contribution to our understanding of pertussis epidemiology. In most cases, the authors have done a thorough job of either addressing or responding to my comments. However, I do not believe the authors engaged sufficiently with two of the queries raised in my previous round of comments. The two queries were about the vaccination status of the mothers and engagement with literature on asymptomatic transmission. I still think they matter and ask the authors to consider them again.

I do not think the authors can rule out two alternative explanations: (1) recent introduction of pertussis and low vaccination coverage amongst study mothers, or (2) recent introduction of a breakthrough strain (either w.r.t. the vaccine or prior infection) and higher vaccination coverage/infection-derived immunity amongst study mothers. Depending on which mechanism was mostly driving the observed patterns in Zambia, i.e.,

- a. long-running, widespread, unreported transmission;
- b. transmission started recently, and vaccination was low amongst study mothers;
- c. a breakthrough strain is causing the current rise (here we'd still want to know about vaccination status); and

d. something else that I have not considered

would have implications for how the results are interpreted, and potentially far-reaching implications for the broader pertussis community. All of that is to say, I think the authors were too quick to dismiss these concerns (even if they disagree with my assertions).

In their reply, the authors largely dismissed concerns about not knowing the mother's vaccination status, stating in their reply that, "our findings strongly suggest ongoing pertussis transmission in this population. Based on this, we expect that mothers in our study who were not vaccinated would likely have some degree of infection-derived immunity."

However, they also stated that, "Zambia offers an evocative example of pertussis surveillance, where no cases have appeared in official WHO reports since 2009" and "As we noted above (and now address in our Discussion), widespread genomic surveillance and microbiological characterization of pertussis are sorely lacking across Africa."

I don't disagree with the authors' conclusion that pertussis is clearly spreading in Zambia. I also don't disagree that there's clearly evidence for minimally symptomatic, infectious mothers spreading infections to children. Both of these findings matter for Zambia and for our broader understanding of pertussis. However, I don't see how the authors can so confidently conclude that low vaccination rates, coupled with a recent introduction, high vaccination rates, coupled with a breakthrough strain, or high infection-derived immunity, coupled with a breakthrough strain, couldn't be what's driving the increase. The authors do hedge in places and also state in the discussion that their findings don't line up with expectations related to WP/infection-derived immunity, "This corresponds to a mean return frequency of one infection per 14.8 years, which is much shorter than the presumed duration of immunity from natural infection or the whole-cell pertussis vaccination used in Zambia (70, 71)." But, my read of the paper is that the authors are pushing way to ward for a preferred hypothesis that is not more favored than other alternatives.

Secondly, I asked about placing this work in the context of other studies on asymptomatic transmission, but realize that I did not list any specific papers. Two worth considering are Warfel et al. 2014 and Althouse and Scarpino 2015. Restating for the editor, the Warfel study found that WP facilitated rapid clearance in a non-human primate experimental infection study (admittedly with small sample sizes and many other caveats). Many took that as evidence that WP would also block transmission (admittedly experiments Warfel did not run). If the mechanism underlying the results in Zambia is that either WP or natural infection does not block transmission (in the absence of a breakthrough strain), that would upend many of the assumptions in pertussis research. While not incompatible with the Warfel et al. results, it would negate most of the importance of their finding that WP blocked transmission. From what I can see, the authors do not even cite Warfel et al. 2014, which is a serious gap regardless of whether the authors agree or disagree with the findings. A quick sidebar, the authors seem to duplicate Craig et al. 2020 10.1093/cid/ciz531, listing it as both citation 9 and 38.

In Althouse and Scarpino, they found evidence of a rise in asymptomatic/underreported/subclinical transmission following the switch from WP to AP. While not as directly relevant to the current study as the Warfel paper (so I leave it to the authors to decide whether citing this paper is important), Althouse and Scarpino discuss asymptomatic transmission at length and also assume that WP conferred strong protection against transmission, so their results (along with dozens and dozens of other studies assuming similar WP/infection-induced immunity protection and durability) would also need to be reinterpreted in the context of this study. The authors should engage with the implication of their results in the context of past modeling studies and what we think we know about vaccine-/infection-derived immunity.

Going back to my earlier points, unvaccinated mothers and the recent introduction of pertussis, or vaccinated/infection-induced immune mothers with a breakthrough strain, would both explain the current results and be compatible with Warfel et al., Althouse and Scarpino, and a sizable number of other studies. Instead, if transmission from WP- or naturally infected mothers is common (in the absence of a breakthrough strain), that would really change the landscape of pertussis epidemiology. The authors have not convinced me that they can make this conclusion. Hence, why I think it's important that the authors engage more actively with those hypotheses and with relevant debates in the pertussis literature on asymptomatic transmission. I think it's appropriate for the authors to present their preferred hypothesis, but, absent other data, they should also present plausible alternatives that are consistent with past publications.

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Author response:

The following is the authors' response to the current reviews.

We again thank the editor and reviewers for their detailed attention to our work. In our previous revisions we endeavored to address the principal concerns raised by reviewers that we were capable of addressing. We recognize that asymptomatic pertussis transmission represents a particularly thorny area of epidemiology and public health, where multiple (and sometimes overlapping) mechanisms have been proffered even as empirical evidence remains thin, particularly in low-resource settings such as sub-Saharan Africa. A key finding of our work is that prospective surveillance in such a low-resource setting revealed abundant evidence of otherwise unobserved asymptomatic incidence. Moreover, as we note in our prior revisions, this finding is supported by recent work in South Africa and elsewhere (Kayina et al., 2015; Moosa et al., 2019, 2025). As such, we believe that further prospective surveillance in similar settings would be highly informative, a point that we have sought to emphasize in our present manuscript (and associated commentary).

While we broadly agree with many of the concerns raised by the reviewers, we believe that we are unable to significantly strengthen the present work through further revisions. We do, however, wish to respond to several points raised in these reviews. Of note, a reviewer raises the possibility of a "breakthrough strain" without reference to existing literature. We agree that we cannot test this hypothesis, and though it is not incompatible with our own findings, it is, however, not consistent with recent molecular surveillance in South Africa (Moosa et al., 2023). The reviewer also raises the potential of low adult vaccination coupled with recent reintroduction. This hypothesis relies on our investigation looking "at the right place and the right time", and further does not explain how immunologically naive adults would have escaped morbidity. We have adopted what we believe is a more parsimonious interpretation of our results (i.e., that asymptomatic infection represents evidence of previous immune exposure), though we agree that a more thorough exploration of this particular issue is warranted, particularly in light of our persistently colonized mothers. In addition, we have noted similar studies in sub-Saharan Africa that *also* found widespread evidence of asymptomatic pertussis, which we believe is inconsistent with a "right time, right place" interpretation.

A reviewer also pointed to the work of Warfel et al. (2014) and Althouse and Scarpino (2015). We are familiar with both of these studies and agree with their broad relevance to the field (our apologies for omitting Warfel et al.). The reviewer states that, "If the mechanism underlying the results in Zambia is that either WP or natural infection does not block transmission (in the absence of a breakthrough strain), that would upend many of the assumptions in pertussis research." Critically, we believe that our prospective field study of human patients in a real-world public health system complements previous research, including animal trials and simulation studies. Simply put, given that our study was unable to establish the prior vaccination or exposure status of participants, we do not claim to have shown evidence for transmission despite wP vaccination or prior infection.

Regarding Althouse & Scarpino (2015), we believe that, for the majority of readers, the most compelling analysis in their paper was the examination of genome sequences that pointed to substantial asymptomatic transmission in the US. This conclusion emerged from their population model, which required that "births" (representing transmission events) exceeded "deaths" (representing recovery of infectious individuals) in order to be consistent with the sequence data. Unfortunately, this paper does not provide a detailed explanation of their methods and data sources, nor is this work directly reproducible through, for example, an open-access code/data repository. We have explored the availability of US genome sequences over the time period of their study and were able to find only 36 sequences: 2 from the pre-vaccine era, 8 from the wP vaccine era, and 26 from the aP vaccine era. Given this notable imbalance in the number of sequences (and thus sequence diversity) that was biased in favour of the most recent time period, is it then surprising that the "birth rate" in their model had to exceed the "death rate" in order to match the genetic diversity in the data? Based on a careful inspection of this work, we do not consider its conclusions to represent a gold standard against which all subsequent studies should be judged. We also note that genomic surveillance and analysis of pertussis remains sparse relative to other fields, though recent works have added dramatically to the corpus of available sequences (Bridel et al., 2022).

Finally, we note that our previous revisions addressed several concerns raised in the present reviews. For example, we previously sought to address reviewers' about our presentation of the strength of our evidence. In this regard, we broadly agree with the reviewers, and we now state that our results "suggest that pertussis transmission occurs between minimally symptomatic mothers and their newborn infants." We believe this largely addresses a present reviewer's concern that 'the mother-to-infant transmission pathway should be framed as "highly suggestive" rather than "confirmed"'. We also note that our results examine three different threshold Ct values (survival analysis, Fig 4), a point that we believe partially addresses a reviewer's suggestion to "including a sensitivity analysis using a stricter cut-off" and concerns about "the decision to use a Ct<45 threshold, as this is higher than standard clinical cut-offs". Indeed, we discuss the issue of clinical cut-offs (and their appropriateness) at some length in the section, "Test reliability, disease surveillance, and public health where we state, "We recognize that such weak and potentially ambiguous signals may not be appropriate for clinical diagnosis. However, our results demonstrate that they nonetheless contain valuable information about pathogen presence and infection intensity that can (and should) be leveraged for disease surveillance." We have also included in the present work a detailed discussion of qPCR sensitivity and efficiency that we believe should interest others working in pertussis surveillance.

We do not view our own research as the "last word" in this rather controversial subject. In this spirit, we have attempted to present our work transparently, state our claims carefully, and underscore future activities that we believe would benefit the pertussis research community going forward. For example, we agree that further attention to shared exposure and functional immune data among low-resource communities could provide valuable insights into epidemiology and ecology of pertussis. However, we also believe the trade-offs

of including one set of activities over another should be clearly acknowledged by researchers, clinicians, and public health officials. To simply state that we must measure *more* fails to account for the very real resource constraints that we all face.

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Moosa, F., du Plessis, M., Weigand, M. R., Peng, Y., Mogale, D., de Gouveia, L., Nunes, M. C., Madhi, S. A., Zar, H. J., Reubenson, G., & others. (2023). Genomic characterization of *Bordetella pertussis* in South Africa, 2015–2019. *Microbial Genomics*, 9(12), 001162.

Moosa, F., du Plessis, M., Wolter, N., Carrim, M., Cohen, C., von Mollendorf, C., Walaza, S., Tempia, S., Dawood, H., Variava, E., & others. (2019). Challenges and clinical relevance of molecular detection of *Bordetella pertussis* in South Africa. *BMC Infectious Diseases*, 19, 1–11.

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Warfel, J. M., Zimmerman, L. I., & Merkel, T. J. (2014). Acellular pertussis vaccines protect against disease but fail to prevent infection and transmission in a nonhuman primate model. *Proceedings of the National Academy of Sciences*, 111(2), 787–792. <https://doi.org/10.1073/pnas.1314688110>

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

The study investigates the role of asymptomatic pertussis carriage in transmission between mothers and their infants, in particular. The authors used a longitudinal cohort study that involved 1,315 mother-infant dyads in Lusaka, Zambia, and they utilized qPCR-based detection of IS481 to track Bordetella pertussis transmission over time. Insights from the study suggest that minimally symptomatic or asymptomatic mothers may act as a reservoir for B. pertussis transmission in the infants, thus challenging the traditional surveillance methods that focus on symptomatic cases. Additionally, the study also identified a subgroup of persistently colonized individuals where mothers were majorly asymptomatic despite sustained bacterial presence.

The authors aimed to improve comprehension of pertussis transmission dynamics in high-burden low-resource settings, and they advocated for enhanced molecular surveillance strategies to capture full pertussis infection, including those that might have gone undetected.

Strengths:

The strengths are the use of innovative study design, especially the longitudinal approach and routine sampling, rather than symptom-driven testing that minimizes bias in the study. The methodology was also rigorous and transparent by evaluating the IS481 signal strength to classify pertussis detection and conducting retesting to assess qPCR reliability. There were also important epidemiological insights, and the findings challenge the traditional wisdom by suggesting that pertussis transmission may frequently occur outside of symptomatic cases. The findings also showed its relevance to global health and policy by arguing for the incorporation of molecular tools like qPCR for surveillance of pertussis in low-resource settings.

Weaknesses:

These include reliability on qPCR-based detection without additional validation measures like confirmatory culture or serology. There are also potential alternate explanations for transmission patterns observed in the study such as shared environmental exposure or household transmission. Additionally, there is limited generalizability as the study was done in a single urban site in Zambia. There is also a lack of functional immune data.

Reviewer #2 (Public review):

Summary:

In this paper, the authors describe the results of a longitudinal study of pertussis infection in mother/infant dyads in Lusaka, Zambia. Unlike many past studies, the authors assessed the infection status of individuals independently of whether they were symptomatic for a respiratory infection. As a result, this work represents one of the first studies specifically designed to assess asymptomatic transmission of pertussis. Using qPCR, the authors find strong evidence for the role of asymptomatic transmission from mothers to infants and also evidence for long-term bacterial carriage. This work represents an important contribution to our understanding of the global burden of pertussis. Also, it highlights the still under-appreciated role of asymptomatic transmission across many infectious diseases (including vaccine-preventable ones).

Strengths:

Unlike many past studies, the authors assessed the infection status of individuals independently of whether they were symptomatic for a respiratory infection. As a result, this work represents one of the first studies specifically designed to assess asymptomatic transmission of pertussis. Using qPCR, the authors find strong evidence for the role of asymptomatic transmission from mothers to infants and also evidence for long-term bacterial carriage.

Weaknesses:

While I am quite enthusiastic about the work, I am concerned that a number of likely relevant confounders were not discussed and that the broader implications of their findings were not well grounded in the existing literature. For example, I could not find information on the vaccination status of the mothers in the study. Given the conclusions about asymptomatic transmission and the durability of immunity, it is important to know the vaccination status of the mothers. Moreover, did the authors have other metadata on the mother/infant dyads, e.g., household size, vaccination status of household members, etc.? Given the potential implications of more widespread asymptomatic transmission associated with pertussis infection, I believe the authors should better couch their results in the context of the broader debate around asymptomatic transmission.

We appreciate the reviewers' detailed feedback. We provide an overview of our responses here and we address specific recommendations below. In light of reviewers' comments, we have revised our manuscript in order to improve the clarity of our presentation and to better situate our results within the context of the existing literature. Unfortunately, as the field study has been concluded, many of the reviewers' recommendations are not possible. These include additional testing (i.e., culture or serology) or sequencing. We have updated the manuscript to more clearly indicate our knowledge regarding maternal vaccine status and immunological immunity of study participants. We have also provided a more comprehensive overview of existing pertussis studies, including genomic surveillance and details regarding sub-Saharan Africa and Zambia in particular. Finally, we have revised the formatting of Figure 4 (survival analysis) to more clearly highlight differences between mothers and infants and to better align with the text, and note that the underlying results are unchanged.

A particular concern raised in the reviews that we wish to address is the recommendation of culture- or serology-based tests as "confirmatory". We have revised the manuscript in light of this feedback to better reflect our own position on this matter. We believe these recommendations do not adequately account for important trade-offs between testing sensitivity and specificity that are widely recognized in both clinical practice and epidemiology (Enøe et al., 2000; Florkowski, 2008; Swift et al., 2020). When the results of different testing methodology disagree, rarely is one method, a priori, correct. Rather, the disagreement may point to specific test limitations or important biological questions about the study system.

In the case of pertussis detection, cell culture is recognized for its very low sensitivity, while serological detection is complicated by debate around appropriate threshold levels and time horizons for seroconversion and subsequent decay (Lee et al., 2018; van der Zee et al., 2015). Furthermore, while anti-PT antibodies are a common target of serological detection, these are not reliable correlates of protection (Mills, 2001; Wilk et al., 2019), nor are they reliably generated in response to colonization (de Cellès & Rohani, 2024; Graaf et al., 2020). Overall, the detailed relationship between exposure, carriage, transmissible infection, and the dynamics of anti-PT serology remains poorly characterized (Craig et al., 2020; de Cellès et al., 2025).

While we agree that these are important questions in epidemiology and public health, we nonetheless wish to highlight that there is no "free lunch": each additional test and protocol comes with additional cost and complexity that should be evaluated based on the specific goals of the intended surveillance. In our case, the repeated sampling of longitudinal surveillance serves as a low-cost "confirmatory" testing regime. We disagree that cell culture would have added value to the present study and would not recommend its addition to future studies (primarily due to low sensitivity). While we agree that before-and-after serology of mothers would have added important context to the present study, we nonetheless expect that significant ambiguity would have surrounded any such results (e.g., Moosa et al. (2025)).

One area that we strongly agree warrants further attention is the household dynamics in pertussis transmission, particularly in low-resource settings where crowding is common. In our study we were not able to rule out environmental and/or shared transmission events, though our survival analysis did demonstrate a greater impact of mothers on infants than vice versa, results which suggest a causal mechanistic role. In previous studies we detailed the demographics of household size, number of children, and mothers' age (Gill et al., 2021; Gunning et al., 2020), though we have not conducted formal analyses of these important covariates here. We also note that the code and data are freely available, allowing for others to build on our work.

We believe that future prospective studies are an invaluable tool for directly tracking pertussis disease transmission, including both community and household studies. We have argued here for the value of qPCR-based community surveillance, which could integrate into

existing public health activities. Regarding household studies, we note that a key challenge in implementing these studies is selecting an appropriate sampling interval and duration to best capture epidemiological linkages. Our results suggest that qPCR-based real-time population-level surveillance could be used to initiate such a prospective household study during a pertussis outbreak so that a higher sampling frequency (e.g., weekly swabs) could be gainfully employed over a shorter time period.

Recommendations for the authors:

Reviewer #1 (Recommendations for the authors):

(1) Enhance Validation of qPCR Findings:

We address these comments above at greater length. We also note that the term “false positive” is rather ambiguous here, as we lack a clear distinction between carriage and transmissible infection. We note that our manuscript includes a considerable discussion of qPCR validation, including negative controls and sample retesting. We agree that test sensitivity and specificity remains an important question, and have endeavored to clearly indicate these concerns throughout the manuscript.

(2) Clarify Transmission Dynamics:

While we agree that this is an important question, we lack the relevant sequence data to test it. Notably, we suspect that any such phylodynamic linkage would require genomic sequencing due to the relatively low genetic diversity observed in pertussis (see, e.g., population-level estimates of time to most common ancestor in Lefranc et al., (2022). However, sequencing pertussis genomes remains resource-intensive, and we expect that the deployment of such sequencing at scale would be cost-prohibitive in low-resource settings.

We have revised our manuscript to underscore uncertainties around shared/household exposure. We also direct the reviewer’s attention to our survival analysis, where a notable asymmetry exists between mothers and infants. Here, mothers’ prior qPCR signals exhibit a larger impact on their infants than infants on their mothers (Fig 4). If shared exposure was the principal cause of the observed increase in hazard in ego from alter, then we would expect no such asymmetry between mothers and infants.

(3) Expand Discussion on Public Health Implications:

As noted above, we have revised our introduction and expanded our discussion to better account for the existing literature. And, while we are hesitant to put forward specific recommendations based on our study, we feel confident in stating that active pertussis surveillance in low-resource settings is A) almost entirely absent B) possible to achieve, and C) necessary to resolve long-standing questions about pertussis epidemiology at regional and national levels. We have endeavored to clarify these points, particularly within the discussion.

(4) Address the Role of Immunity More Directly:

While we lack such immune data, we have pointed to recent work from the notable PHIRST study in South Africa, as well as highlighted ambiguities surrounding these data.

Reviewer #1 (Recommendations for the authors):

(1) Do we know the vaccination status of the mothers in the study? ... If these data are not available, I think that the paper must be re-framed to acknowledge that all the conclusions are statistical in nature, based on publicly available vaccine coverage data from Zambia.

We do not have information on the immunization status of mothers, though we cite national rates for Zambia across the relevant time period. We have clarified this point in the revised manuscript. We have endeavored to clearly acknowledge that many of our conclusions are statistical in nature and to clearly quantify the strength of evidence.

We strongly disagree that anomalously low vaccination rates amongst mothers (i.e., relative to national averages) would materially alter the interpretation of our findings.

Overall, our findings strongly suggest ongoing pertussis transmission in this population. Based on this, we expect that mothers in our study who were not vaccinated would likely have some degree of infection-derived immunity. Indeed, some have argued that the preponderance of mild/asymptomatic infections in mothers is, of itself, evidence of prior immunological exposure (Fine & Clarkson, 1982).

(2) Do we know anything about rates of pertussis in Zambia, especially in the study site?

We address this important question in the discussion. In particular, we state that: “As a populous, middle-income and primarily urban country, Zambia offers an evocative example of pertussis surveillance, where no cases have appeared in official WHO reports since 2009”.

(3) I couldn't find information in the paper related to the severity of infection in the infants. It's mentioned in the section describing results in Figure 5, but I only saw analyses with symptoms (as opposed to severe symptoms). Do you have outcome data from infants testing positive?

This question was addressed in more detail in our previous work (Gill et al., 2021), which we briefly summarize in the Introduction. We also show the frequency of severe symptoms in Fig 5 (bottom panel), and detail mild versus serious symptoms in our subgroup analysis (Fig 7D).

(4) Do we know anything about vaccine-resistant strains of pertussis in Zambia?

We are not aware of any such work. As we noted above (and now address in our Discussion), widespread genomic surveillance and microbiological characterization of pertussis are sorely lacking across Africa.

(5) While I believe sequencing is beyond the scope of the current study, the authors should comment on the potential utility of sequencing elements of the pertussis genome and use that to demonstrate causality and direction of transmission more strongly.

We believe that existing literature has addressed the potential of sequence data and phylodynamics to infer transmission, particularly for pathogens with high mutation rates such as RNA viruses. To date, research into the phylodynamics of pertussis has focused exclusively on population-level dynamics (Lefrancq et al., 2022), where estimates of time to most recent common ancestor (TMRCA) are long, indicating low genomic variability at the scale of countries and years. To our knowledge, no work on pertussis has directly inferred transmission chains from sequence data. Given the existing evidence, we expect that any such work would require genome-level sequencing, which would likely be cost-prohibitive in low-resource settings.

(6) ... However, it would be helpful to understand more about how your results fit into the broader story around pertussis resurgence. ... if the infant cases were all mild, they might never have been captured in surveillance data sets.

We believe that a key result of our study is the remarkable mismatch between country-level symptoms-based surveillance and prospective surveillance, which demonstrates that such mild cases have almost certainly not been captured. These findings are mirrored by recent work in South Africa (now addressed in our Discussion, see Moosa et al. (2025)). We believe that prospective surveillance, particularly in under-surveilled regions, is critical to understanding pertussis transmission writ large, which we have attempted to communicate throughout our discussion.

(7) Relatedly, if there are still high rates of asymptomatic mother-to-infant transmission with whole cell vaccination, then why is there an observed drop in infant pertussis following whole vaccination in most countries?

In previous work, we demonstrated that some infants in this cohort exhibited asymptomatic infection (Gill et al., 2021). We note that a drop in pertussis incidence amongst infants after the roll-out of the whole-cell vaccine is not contradictory with our findings. We want to clarify that our results, and evidence that mother-to-infant transmission can occur, does not imply that the whole-cell vaccine fails to protect against transmission.

We have previously used epidemiological evidence to infer the population-level impacts following the roll-out of whole-cell pertussis infant immunization. For example, we observed an increase in the inter-epidemic period that, together with the drop in infant cases, are consistent with a reduction in transmissible infections (Broutin et al., 2010; Rohani et al., 2000).

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