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## Practice of Epidemiology

### Measurement of Vaccine Direct Effects Under the Test-Negative Design

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Test-negative designs are commonplace in assessments of influenza vaccination effectiveness, estimating this value from the exposure odds ratio of vaccination among individuals treated for acute respiratory illness who test positive for influenza virus infection. This approach is widely believed to recover the vaccine direct effect by correcting for differential health-care-seeking behavior among vaccinated and unvaccinated persons. However, the relationship of the measured odds ratio to true vaccine effectiveness is poorly understood. We derived the odds ratio under circumstances of real-world test-negative studies. The odds ratio recovers the vaccine direct effect when 2 conditions are met: 1) Individuals' vaccination decisions are uncorrelated with exposure or susceptibility to the test-positive or test-negative conditions, and 2) vaccination confers "all-or-nothing" protection (whereby certain individuals have no protection while others are perfectly protected). Biased effect-size estimates arise if either condition is unmet. Such bias might suggest misleading associations of vaccine effectiveness with time since vaccination or the force of infection of influenza. The test-negative design could also fail to correct for differential health-care-seeking behavior among vaccinated and unvaccinated persons without stringent criteria for enrollment and testing. Our findings demonstrate a need to reassess how data from test-negative studies can inform policy decisions.

influenza; test-negative design; vaccine effectiveness

Abbreviations: ARI, acute respiratory illness; OR, odds ratio; VE, vaccine effectiveness or efficacy.

Observational study designs (1, 2) are needed to measure vaccine effectiveness (VE) when randomized trials are infeasible or unethical, as with the new formulations of influenza vaccines used each year (3). The "test-negative" design—a modification of the traditional case-control design—has become popular for measuring clinical effectiveness of seasonal influenza vaccines (1). It resembles earlier designs, such as the indirect cohort method (4) and the selection of "imitation disease" controls in case-control studies (5). Individuals who experience acute respiratory illness (ARI) and present for care receive a laboratory test for influenza virus infection, and their vaccination history is ascertained. The exposure odds ratio (OR) of vaccination among test-positive and test-negative subjects, in some instances adjusted for potential confounding using stratification or regression, has frequently been used to measure VE (6), where  $VE = (1 - OR) \times 100\%$  (7). Causal interpretations of resulting estimates have become the basis for policy making, such as the US Advisory Committee on Immunization Practices recommendation that quadrivalent live attenuated influenza

vaccine should not be used in the US during the 2016–2017 and 2017–2018 seasons (3, 8, 9).

Unlike VE estimates from traditional case-control studies, the test-negative measure is expected to correct for differential treatment-seeking behaviors among vaccinated and unvaccinated persons because only individuals who seek care are included (10). However, potential confounding, misclassification, and selection biases under the test-negative design (2, 11–13) have ignited debate about the suitability of test-negative studies as a basis for policymaking. Whereas directed acyclic graphs have been useful in revealing such biases (9, 14, 15), quantitative implications of these biases for VE estimates remain uncertain (16).

To resolve this uncertainty, we derived the relationship of the test-negative odds ratio to true VE, defined as the vaccine-conferred reduction in susceptibility to influenza infection and/or influenza-caused ARI (vaccine "direct effect" (17)). We used this mathematical relationship to assess the quantitative impact of potential biases in test-negative studies. We considered a test-negative study of VE against seasonal influenza

as a guiding example, noting that our findings have implications for test-negative studies of vaccines against rotavirus (18, 19), cholera (20, 21), meningococcus (22), pneumococcus (4), and other infections.

**NOTATION**

For consistency, we used notation from a previous study (10) where possible; we list all parameters and definitions in Table 1. We assumed that ARI could result from influenza infection (*I*) or other causes (*N*). Susceptible individuals acquire infection at time-constant rates  $\lambda_I$  and  $\lambda_N$ ; we show later that results hold for seasonal or otherwise time-varying acquisition rates  $\lambda(t)$ . We defined  $t = 0$  as the start of the influenza season and assumed individuals were vaccinated around this time (before extensive transmission). Infections cause ARI with probability  $\pi_I$  and  $\pi_N$ , respectively. Out of the entire population *P*, a proportion of individuals (*v*) receive vaccine. Because individuals who opted for vaccination might differ from others in their likelihood for seeking treatment for ARI, we defined the probability of seeking treatment for an ARI episode as  $\mu_V$  among the vaccinated and  $\mu_U$  among the unvaccinated; we address how differential treatment seeking for test-positive and test-negative conditions influences estimates in a later section.

Because a single type or subtype of influenza typically dominates each season, we assumed that naturally acquired immunity protects against within-season reacquisition of influenza. The proportion of individuals remaining susceptible to infection at time *t* is thus  $e^{-\lambda t}$ . We assumed further that the various non-influenza causes of ARI (*N*) are unlikely to provide immunity against one another, so that the full population remains at risk of *N* throughout; we show later that this assumption does not affect estimates (Web Appendix 1, available at <https://academic.oup.com/aje>).

We considered 2 mechanisms by which vaccination protects against infection. We defined  $\phi$  as the proportion of individuals

responding to vaccine, so that a proportion  $1 - \phi$  remains unaffected by vaccination; here we assumed individuals' likelihood of responding was unassociated with exposure or susceptibility to infection. Among the responders, we defined  $\theta$  as the hazard ratio for infection (measured relative to the hazard rate of infection among nonresponders and unvaccinated persons) resulting from vaccine-derived protection (23, 24). The special case where  $\theta = 0$  and  $0 < \phi < 1$  corresponds to a situation of "all-or-nothing" protection for responders and nonresponders, respectively, while "leaky" protection for all recipients arises under  $\phi = 1$  and  $0 < \theta < 1$  (17, 23, 24), whereby all vaccine recipients experience a reduced rate of acquiring infection. We note that this definition of "leaky" protection is unrelated to the relative risk for vaccine recipients and nonrecipients to experience progression of infection to symptomatic disease (17), and we consider this issue in a subsequent section. The general circumstances of  $0 < \phi < 1$  and  $0 < \theta < 1$  correspond to an intermediate scenario of "leaky-or-nothing" protection. Perfect protection attains for  $\theta = 0$  and  $\phi = 1$ , and no protection attains when  $\phi = 0$  (no individuals respond to vaccination) or  $\theta = 1$  (responders receive no protection). The vaccine direct effect on susceptibility to infection is the rate ratio of infection given vaccination:

$$VE = 1 - [(1 - \phi) + \theta\phi] = \phi(1 - \theta).$$

This parameter is of interest in vaccine studies as the basis for calculating the effective reproductive number and the critical population to vaccinate (25). To highlight design-level features most pertinent to the interpretation of test-negative studies, and in line with typical reporting of VE estimates, our analysis does not address heterogeneity in vaccine response beyond the consideration of "all-or-nothing" and "leaky-or-nothing" protection, nor do we address impacts of vaccination on infectiousness, given that estimates from conventional test-negative studies do not capture indirect effects. We refer readers to previous studies addressing such issues in the contexts of differing study designs (17, 26–28). Where applicable, we addressed sources of confounding in test-negative studies that could lead to incorrect inferences of heterogeneity in vaccine effects among individuals or over time.

**Table 1.** Parameters Describing the Incidence of Test-Positive and Test-Negative Diagnoses, Referenced in Order of Appearance

Parameter	Definition
$\lambda$	Force of infection (baseline rate of infection acquisition per susceptible)
$\pi$	Probability of ARI given infection
<i>P</i>	Total population
<i>v</i>	Proportion of the population vaccinated
$\mu$	Probability of seeking treatment given ARI
$\phi$	Proportion of individuals responding to vaccine
$\theta$	Hazard ratio for infection resulting from vaccine-derived protection (among responders)
$\alpha$	Hazard ratio for infection (relative to population average) due to factors other than vaccine-derived protection
$\xi$	Probability of laboratory diagnostic testing given health-care seeking for ARI
$\rho$	Relative risk of ARI given infection due to vaccine-derived protection

Abbreviation: ARI, acute respiratory illness.

**PERFORMANCE OF THE ODDS RATIO UNDER VACCINATION UNCONFOUNDED BY EXPOSURE OR SUSCEPTIBILITY TO THE CONDITIONS**

Here we considered the case where individuals' decision-making about whether to receive influenza vaccine is uncorrelated with their a priori risk of acquiring influenza and test-negative conditions and with the probability that these conditions would cause ARI (or another clinical endpoint of interest for study enrollment;  $\pi_I$  and  $\pi_N$ ). To examine the potential for the test-negative design to correct for treatment-seeking biases, we allowed vaccine recipients and nonrecipients to have different probabilities of seeking treatment for ARI ( $\mu_V$  and  $\mu_U$ ), assuming for now that these probabilities are unaffected by the cause of the ARI. We relax this assumption in a later section.

To understand what the odds ratio measures in test-negative studies, we derived the rate at which individuals enter into the study

as test-positive or test-negative subjects given their vaccination status. The rate of ascertaining test-positive, vaccinated persons is

$$\Lambda_{VI}(t) = \lambda_I \pi_I \mu_V [(1 - \varphi)e^{-\lambda_I t} + \varphi \theta e^{-\theta \lambda_I t}] \nu P,$$

where the force of infection ( $\lambda_I$ ) is applied upon as-yet-uninfected members of the vaccinated population; we further account for the proportion ( $\pi_I \mu_V$ ) of individuals expected to show symptoms and seek treatment. The rate of ascertaining test-positive, unvaccinated subjects is

$$\Lambda_{UI}(t) = \lambda_I \pi_I \mu_U e^{-\lambda_I t} (1 - \nu) P.$$

Test-negative vaccinated and unvaccinated persons are ascertained at the rates

$$\Lambda_{VN}(t) = \lambda_N \pi_N \mu_V \nu P$$

and

$$\Lambda_{UN}(t) = \lambda_N \pi_N \mu_U (1 - \nu) P,$$

respectively, assuming vaccination does not affect susceptibility to the test-negative conditions.

Test-negative studies typically measure the odds ratio of vaccination among the test-positive and test-negative subjects, similar to the exposure odds ratio in case-control studies, using cumulative cases ( $C$ ). For the test-positive outcome,

$$C_{VI}(t) = \pi_I \mu_V [(1 - \varphi)(1 - e^{-\lambda_I t}) + \varphi(1 - e^{-\theta \lambda_I t})] \nu P$$

$$C_{UI}(t) = \pi_I \mu_U (1 - e^{-\lambda_I t}) (1 - \nu) P.$$

Under the assumption that test-negative infections are not immunizing, cumulative cases are proportional to the incidence rate and study duration:

$$C_{VN}(t) = \lambda_N \pi_N \mu_V \nu P t$$

$$C_{UN}(t) = \lambda_N \pi_N \mu_U (1 - \nu) P t.$$

We consider the case of immunizing test-negative outcomes in Web Appendix 1. Using the vaccine-exposure odds ratio measured from cumulative cases,

$$\begin{aligned} 1 - OR^C(t) &= 1 - \frac{C_{VI}(t)C_{UN}(t)}{C_{UI}(t)C_{VN}(t)} \\ &= 1 - \frac{(1 - \varphi)(1 - e^{-\lambda_I t}) + \varphi(1 - e^{-\theta \lambda_I t})}{1 - e^{-\lambda_I t}} \\ &= \varphi \left( 1 - \frac{1 - e^{-\theta \lambda_I t}}{1 - e^{-\lambda_I t}} \right). \end{aligned} \tag{1a}$$

Under the special case of “all-or-nothing protection” ( $\theta = 0$ ),

$$1 - OR^C(t) = \varphi,$$

equal to the vaccine direct effect against infection. In contrast, under the special case of “leaky” protection for all recipients ( $\varphi = 1$ ),

$$1 - OR^C(t) = 1 - \frac{1 - e^{-\theta \lambda_I t}}{1 - e^{-\lambda_I t}},$$

resulting in a bias toward the null value of 0. This bias is non-existent near  $t = 0$  ( $\lim_{t \rightarrow 0} [1 - OR^C(t)] = \theta$ ), but grows as  $t$  increases ( $\lim_{t \rightarrow \infty} [1 - OR^C(t)] = 0$ ).

Despite the lack of data in test-negative studies on the population (or person-time) at risk for infection, this result (equation 1a) is equal to VE measures from the relative risk in randomized controlled trials. While methods have previously been proposed to recover the vaccine effect on susceptibility through uses of population-at-risk or person-time-at-risk data (23, 29), we note that the absence of such measurements presents a unique obstacle to bias correction in test-negative studies.

Studies can also measure time-specific odds ratios, for instance by stratifying analyses into subseasonal intervals (30–33) or by allowing vaccination and time to interact in logistic regression models fitted to individual-level data (34, 35). In comparison with odds ratios estimated from cumulative cases, such estimates are often sought to gauge differences over time in VE, for instance due to waning of protection. As the time increment approaches zero, terms included in the odds ratio approach the ascertainment rates of test-positive and test-negative subjects. We therefore define this measurement as

$$\begin{aligned} 1 - OR^\Lambda(t) &= 1 - \frac{\Lambda_{VI}(t)\Lambda_{UN}(t)}{\Lambda_{UI}(t)\Lambda_{VN}(t)} \\ &= 1 - [(1 - \varphi) + \varphi \theta e^{-\lambda_I t (\theta - 1)}] \\ &= \varphi (1 - \theta e^{-\lambda_I t (\theta - 1)}), \end{aligned} \tag{1b}$$

again reducing to

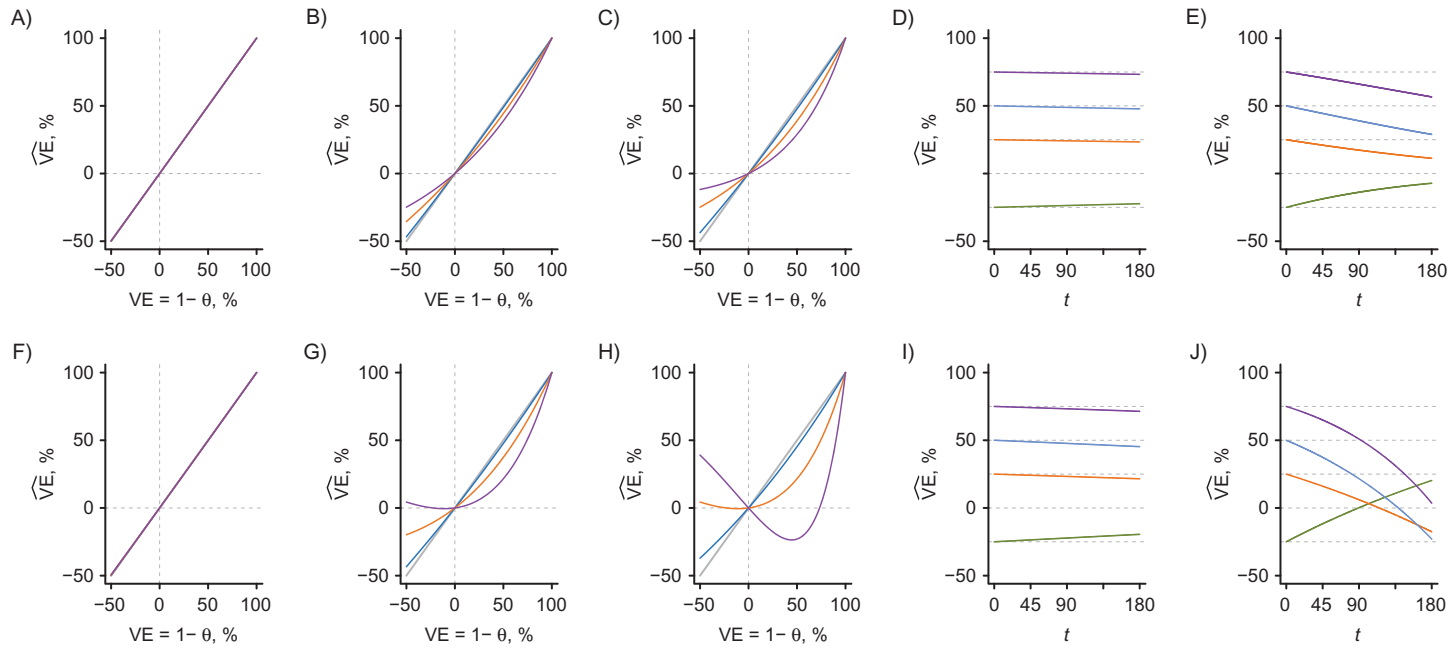
$$1 - OR^\Lambda(t) = \varphi$$

under “all-or-nothing” protection but allowing bias to persist under “leaky” protection for all recipients:

$$1 - OR^\Lambda(t) = 1 - \theta e^{-\lambda_I t (\theta - 1)}.$$

Here bias is again nonexistent at  $t = 0$  and worsens as  $t \rightarrow \infty$ , further increasing with  $\lambda_I$ . Intuitively, the bias arises due to differential depletion of vaccinated and unvaccinated susceptible individuals, consistent with other study designs (23, 36, 37). Presuming the vaccine is efficacious, more unvaccinated than vaccinated individuals will have been depleted later in the epidemic, confounding instantaneous comparisons.

We illustrate functional forms of  $1 - OR^C(t)$  and  $1 - OR^\Lambda(t)$  under scenarios of “leaky” and “leaky-or-nothing” protection in Figures 1 and 2, respectively. Considering first a “leaky” vaccine with  $1 - \theta = 50\%$  efficacy, under conditions of  $\lambda_I = 0.001, 0.005,$  and  $0.01$  infections/person-day, VE estimates based on cumulative cases are 2.2%, 11.2%, and 22.1% lower than the true vaccine efficacy, respectively, after 90 days of influenza transmission (Figure 1); by this point, 8.6%, 36.7%, and 59.3% of unvaccinated individuals are expected to have been infected. Serological studies have revealed cumulative infection rates in the range of 20%–40% for seasonal influenza (38, 39) and up to 47% for influenza A(H1N1)pdm09 (40)



**Figure 1.** Test-negative measures under “leaky” protection. We illustrate test-negative vaccine efficacy (VE) estimates obtained from the exposure odds ratio (OR) for a vaccine conferring “leaky” protection ( $\varphi = 1$ ) to all recipients; Figure 2 includes extensions to “leaky-or-nothing” protection with differing values of  $\varphi$ . Estimates use cumulative case data ( $1 - OR^C$ ) (panels A–E) and ascertainment rates ( $1 - OR^A$ ) (panels F–J), under an assumption of no correlation between vaccination and exposure or susceptibility. Panels A–C and F–H illustrate measurements at set times ( $t$ ) under differing transmission intensity ( $\lambda_t$  equal to rates of 0.001, 0.005, and 0.01 infections per susceptible day at risk for blue, orange, and purple lines, respectively). A) and F) Measurement at  $t = 1$  day. B) and G) Measurement at  $t = 90$  days. C) and H) Measurement at  $t = 180$  days. Panels D, E, I, and J illustrate changes over time in estimated vaccine effectiveness, under scenarios of vaccine effectiveness equal to –25%, 25%, 50%, and 75% for green, orange, blue, and purple lines, respectively. Dashed grey lines are plotted for reference to true effect size measures. D) and I) Estimates under  $\lambda_t = 0.001$  infections per susceptible day at risk. E) and J) Estimates under  $\lambda_t = 0.01$  infections per susceptible day at risk.

among unvaccinated (and presumably susceptible) children, suggesting that reported VE estimates might fall in the middle of this range in terms of bias; differences in susceptibility across ages and risk strata could, however, result in differential rates of infection and differential degrees of bias in estimates (41). The exposure-dependent biases we identify worsen with lower vaccine efficacy: For  $1 - \theta = 20\%$ , estimated values fall 3.6%, 17.1%, and 32.4% below the true effect for  $\lambda_I = 0.001, 0.005,$  and  $0.01$ , respectively. Estimates based on the ascertainment rate ( $1 - OR^\Lambda(t)$ ) would show greater bias at the same point in time: VE estimates are reduced by 4.6%, 25.2%, and 56.8% for a vaccine conferring 50% efficacy, and by 7.3%, 37.7%, and 78.9% for a vaccine conferring 20% efficacy.

Figure 2 illustrates how bias is further influenced by the contributions of vaccine response probabilities to overall vaccine efficacy. For a vaccine conferring 50% efficacy based on 90% of individuals responding (so that  $\theta = \frac{[\varphi - 0.5]}{\varphi} = 44.4\%$ ), and again defining  $\lambda_I = 0.001, 0.005,$  and  $0.01$  infections/person-day,  $1 - OR^C(t)$  as of  $t = 90$  yields values subject to 2.0%, 10.0%, and 20.0% downward bias, respectively. With the same efficacy based on 60% of individuals responding ( $\theta = 16.7\%$ ), the degree of bias is reduced to 0.8%, 3.9% and 8.2% below the true effect.

To aid interpretation in the context of previous studies (2, 11), we have also illustrated the modeled causal process using a directed acyclic graph (Figure 3), revealing that the special case of “all-or-nothing” protection precludes bias from vaccine-derived protection against influenza infections occurring before the ARI episode for which an individual seeks care. We derive VE estimators accounting for additional real-world circumstances—including time-varying transmission intensity during an influenza season and the use of naturally immunizing test-negative endpoints—in Web Appendix 1, showing that the odds ratio retains the biases identified under our simpler initial assumptions.

In some applications, testing for a protective or harmful effect of the vaccine might take priority over obtaining precise measurements of effect size. The conclusions of such hypothesis tests rest on an assumption that the odds ratio is not subject to sign bias, reflecting the circumstance  $OR(t) > 1$  for an effective vaccine (as defined by the condition  $\theta < 1$ ), or  $OR(t) < 1$  for an ineffective vaccine (for which  $\theta > 1$ ). The plots of  $1 - OR^\Lambda(t)$  in Figures 1 and 2 illustrate that VE estimates based on the ascertainment rate of cases might encounter sign bias. The odds ratio measured from ascertainment rates reaches one—suggesting no vaccine effect—when the cumulative transmission to which a population has been exposed ( $\lambda_I \times t$ ) reaches a particular threshold:

$$\lambda_I t = \frac{\ln(\theta)}{\theta - 1};$$

we derive this threshold in Web Appendix 2. Once at least this proportion of the unvaccinated population has become immune to infection, cases will appear with higher frequency among vaccinated individuals than among unvaccinated individuals even when vaccine-derived protection does not wane. These circumstances demonstrate the need for caution in interpreting time-specific (continuous or subseasonal) VE measurements from test-negative studies (30–33), or for strategies to account for previous infection prevalence among vaccinated and unvaccinated persons.

### PERFORMANCE OF THE ODDS RATIO UNDER DIFFERENTIAL EXPOSURE OR SUSCEPTIBILITY OF VACCINATED AND UNVACCINATED PERSONS TO THE CONDITIONS

The test-negative design is typically employed in observational studies where individuals have received vaccination voluntarily. In contrast to assumptions in the above section that vaccination is uncorrelated with exposure or susceptibility to infection, variation in vaccine uptake across risk groups is well-recognized (2). For instance, preferential vaccine receipt has been reported among relatively healthy older adults (42, 43) and among persons prioritized for vaccination such as health-care workers (who might have elevated risk of encountering infected persons) and individuals with underlying health conditions (who might be at risk for severe outcomes if infected) (44, 45). This circumstance corresponds to the presence of a confounder (“G” in Figure 3) related to disease risk as well as vaccination.

Absent vaccine-derived protection, we defined  $\alpha_{VI}/\alpha_{UI}$  and  $\alpha_{VN}/\alpha_{UN}$  as the relative rates at which individuals who seek vaccination would be expected to acquire influenza and test-negative conditions, respectively, measured against the expected rates among individuals who do not seek vaccination. These relative rates do not consider the biological effect of the vaccine but only the counterfactual associated with vaccine-seeking status.

Accounting further for vaccine-induced protection, the ascertainment rates of test-positive and test-negative subjects are

$$\Lambda_{VI}(t) = \alpha_{VI}\lambda_I\pi_I\mu_V[(1 - \varphi)e^{-\alpha_{VI}\lambda_I t} + \varphi\theta e^{-\theta\alpha_{VI}\lambda_I t}]vP$$

$$\Lambda_{UI}(t) = \alpha_{UI}\lambda_I\pi_I\mu_U e^{-\alpha_{UI}\lambda_I t}(1 - v)P$$

$$\Lambda_{VN}(t) = \alpha_{VN}\lambda_N\pi_N\mu_V vP$$

$$\Lambda_{UN}(t) = \alpha_{UN}\lambda_N\pi_N\mu_U(1 - v)P,$$

resulting in cumulative case measures

$$C_{VI}(t) = \pi_I\mu_V[(1 - \varphi)(1 - e^{-\alpha_{VI}\lambda_I t}) + \varphi(1 - e^{-\alpha_{VI}\theta\lambda_I t})]vPt$$

$$C_{UI}(t) = \pi_I\mu_U(1 - e^{-\alpha_{UI}\lambda_I t})(1 - v)Pt$$

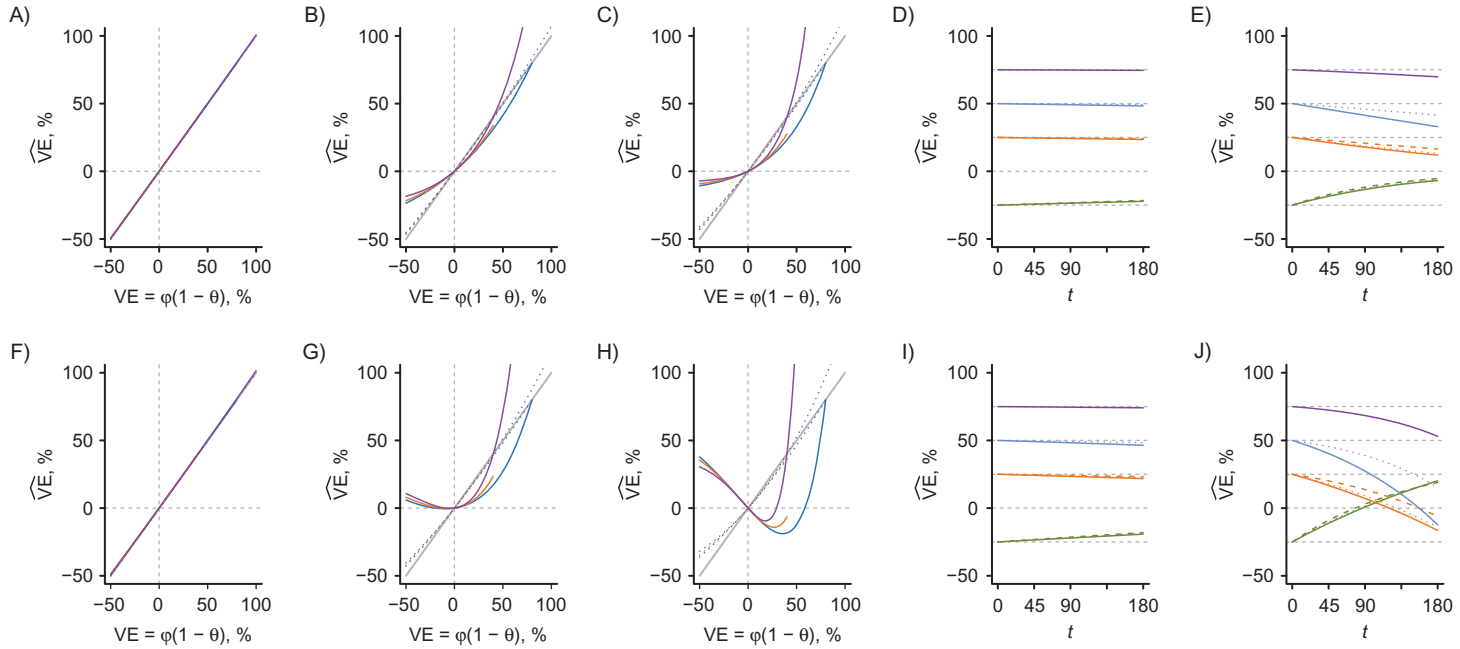
$$C_{VN}(t) = \alpha_{VN}\lambda_N\pi_N\mu_V vPt$$

$$C_{UN}(t) = \alpha_{UN}\lambda_N\pi_N\mu_U(1 - v)Pt.$$

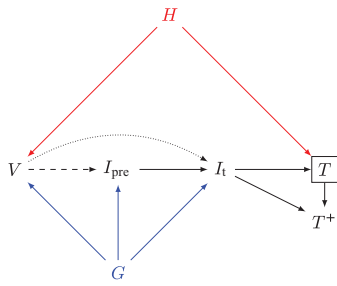
Estimating VE from cumulative cases,

$$\begin{aligned} 1 - OR^C(t) &= 1 - \frac{C_{VI}(t)C_{UN}(t)}{C_{UI}(t)C_{VN}(t)} \\ &= 1 - \frac{\alpha_{UN}}{\alpha_{VN}} \left( \frac{(1 - \varphi)(1 - e^{-\alpha_{VI}\lambda_I t}) + \varphi(1 - e^{-\alpha_{VI}\theta\lambda_I t})}{1 - e^{-\alpha_{UI}\lambda_I t}} \right), \end{aligned} \quad (2a)$$

whereas the estimate based on ascertainment rates is



**Figure 2.** Test-negative measures under “leaky-or-nothing” protection. We illustrate test-negative vaccine efficacy (VE) estimates obtained from the exposure odds ratio (OR) for a vaccine conferring “leaky-or-nothing” protection; compare against Figure 1 for the special case of “leaky” protection ( $\phi = 1$ ). Estimates use cumulative case data ( $1 - OR^C$ ) (panels A–E) and ascertainment rates ( $1 - OR^A$ ) (panels F–J), under an assumption of no correlation between vaccination and exposure or susceptibility. Panels A–C and F–H illustrate measurements at set times ( $t$ ) under differing transmission intensity ( $\lambda_1$  equal to rates of 0.001 and 0.01 infections per susceptible day at risk for dotted and solid lines, respectively); we illustrate performance of the estimator with differing degrees of vaccine response, illustrating  $\phi$  equal to 0.8, 0.6, and 0.4 for blue, orange, and purple lines, and  $\theta = (\phi - VE) \phi - 1$ . A) and F) Measurement at  $t = 1$  day. B) and G) Measurement at  $t = 90$  days. C) and H) Measurement at  $t = 180$  days. Panels D, E, I, and J illustrate changes over time in estimated vaccine effectiveness. As in Figure 1, green, orange, blue, and purple lines signify scenarios of –25%, 25%, 50%, and 75% vaccine effectiveness; dashed, dotted, and solid lines signify  $\phi$  equal to 0.4, 0.6, and 0.8, respectively. Dashed grey lines are plotted for reference to true effect size measures. D) and I) Estimates under  $\lambda_1 = 0.001$  infections per susceptible day at risk. E) and J) Estimates under  $\lambda_1 = 0.01$  infections per susceptible day at risk.



**Figure 3.** Causal directed acyclic graph illustrating a key source of bias for leaky vaccines. Health-care-seeking ( $H$ ) drives receipt of the vaccine ( $V$ ) as well as receipt of a test ( $T$ ). By design, studies select on testing, given that only tested individuals are included. The effect of interest, signified by the dotted arrow, is that of vaccination on influenza at the time of testing ( $I_t$ ). However, influenza might also occur at a preceding point in the season ( $I_{pre}$ , dashed arrow). The test-positive outcome ( $T^+$ ) arises when an individual is infected at the time of testing ( $I_t \rightarrow T^+$ ). Natural immunity prevents influenza reinfection during the season ( $I_{pre} \rightarrow I_t$ ). The fact that  $I_{pre}$  is not—and cannot be—conditioned on leads to a second pathway not of direct interest ( $V \rightarrow I_{pre} \rightarrow I_t$ ), biasing the estimate of the direct effect  $V \rightarrow I_t$  in the case of leaky vaccine. This bias is not present in the case of all-or-nothing protection. Here, 2 distinct subgraphs can be envisioned. In the first—applicable only to the proportion ( $\phi$ ) of protected, vaccinated individuals—the path  $V \rightarrow I_{pre} \rightarrow I_t$  is not of concern, because  $\Pr[I_{pre}|V] = 0$ . In the second, applying to the remaining proportion  $(1 - \phi)$  of unprotected individuals, the paths  $V \rightarrow I_{pre} \rightarrow I_t$  and  $V \rightarrow I_t$  are null, consistent with the situation where  $V = 0$ . Subsequent sections of this manuscript focus on other sources of bias evident in this graph. The first concerns the impacts of a confounder ( $G$ ) of exposure or susceptibility to influenza infection (here indicated in blue; see also Figure 3 of Lipsitch et al. (2)). The second concerns selection bias resulting from differential health-care-seeking behavior among vaccinated and unvaccinated persons along the pathway  $V \leftarrow H \rightarrow T$  (here highlighted in red).

$$\begin{aligned}
 1 - OR^\Lambda(t) &= 1 - \frac{\Lambda_{VI}(t)\Lambda_{UN}(t)}{\Lambda_{UI}(t)\Lambda_{VN}(t)} \\
 &= 1 - \left( \frac{\alpha_{VI} \alpha_{UN}}{\alpha_{UI} \alpha_{VN}} \right) [(1 - \phi)e^{-\lambda_{I,t}(\alpha_{VI} - \alpha_{UI})} \\
 &\quad + \phi\theta e^{-\lambda_{I,t}(\alpha_{VI}\theta - \alpha_{UI})}]. \tag{2b}
 \end{aligned}$$

These estimates reduce to

$$1 - OR^C(t) = 1 - (1 - \phi) \frac{\alpha_{UN}}{\alpha_{VN}} \left( \frac{1 - e^{-\alpha_{VI}\lambda_{I,t}}}{1 - e^{-\alpha_{UI}\lambda_{I,t}}} \right)$$

$$1 - OR^\Lambda(t) = 1 - (1 - \phi) \left( \frac{\alpha_{VI} \alpha_{UN}}{\alpha_{UI} \alpha_{VN}} \right) e^{-\lambda_{I,t}(\alpha_{VI} - \alpha_{UI})}$$

under “all-or-nothing” protection and

$$1 - OR^C(t) = 1 - \frac{\alpha_{VN}}{\alpha_{UN}} \left( \frac{1 - e^{-\alpha_{VI}\theta\lambda_{I,t}}}{1 - e^{-\alpha_{UI}\lambda_{I,t}}} \right)$$

$$1 - OR^\Lambda(t) = 1 - \theta \left( \frac{\alpha_{VI} \alpha_{UN}}{\alpha_{UI} \alpha_{VN}} \right) e^{-\lambda_{I,t}(\alpha_{VI}\theta - \alpha_{UI})}$$

under “leaky” protection.

Consider alternatively that  $\pi_{VI}/\pi_{UI}$  and  $\pi_{VN}/\pi_{UN}$  are the relative risks of ARI given influenza and test-negative infections, respectively, for individuals who seek vaccination, measured against the risk among individuals who do not seek vaccination; we again distinguish that these differences owe to factors other than vaccine-derived protection (17), and we consider vaccine protection against disease progression in a subsequent section. Incorporating  $\pi_I$  and  $\pi_N$  into the odds ratios formulated above,

$$1 - OR^C(t) = 1 - \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) \left( (1 - \phi) + \phi \frac{1 - e^{-\theta\lambda_{I,t}}}{1 - e^{-\lambda_{I,t}}} \right) \tag{3a}$$

and

$$1 - OR^\Lambda(t) = 1 - \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) [1 - \phi(1 - \theta e^{-\lambda_{I,t}(\theta - 1)})]. \tag{3b}$$

Under “all-or-nothing” protection,

$$1 - OR^C(t) = 1 - OR^\Lambda = 1 - (1 - \phi) \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right),$$

reducing to  $\phi$  if differences between vaccinated and unvaccinated persons equally affect progression of influenza and test-negative conditions to symptoms (i.e.,  $\frac{\pi_{VI}}{\pi_{UI}} = \frac{\pi_{VN}}{\pi_{UN}}$ ). For a vaccine conferring “leaky” protection to all recipients,

$$1 - OR^C(t) = 1 - \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) \left( \frac{1 - e^{-\theta\lambda_{I,t}}}{1 - e^{-\lambda_{I,t}}} \right)$$

and

$$1 - OR^\Lambda(t) = 1 - \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) e^{-\lambda_{I,t}(\theta - 1)},$$

reducing when  $\frac{\pi_{VI}}{\pi_{UI}} = \frac{\pi_{VN}}{\pi_{UN}}$  to the bias present when vaccine-seeking is uncorrelated with exposure or susceptibility to infection (equations 1a and 1b).

Incorporating heterogeneity in both acquisition and progression,

$$\begin{aligned}
 1 - OR^C(t) &= 1 - \frac{\alpha_{UN}}{\alpha_{VN}} \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) \\
 &\quad \times \left( \frac{(1 - \phi)(1 - e^{-\alpha_{VI}\lambda_{I,t}}) + \phi(1 - e^{-\alpha_{VI}\theta\lambda_{I,t}})}{1 - e^{-\alpha_{UI}\lambda_{I,t}}} \right) \tag{4a}
 \end{aligned}$$

and

$$\begin{aligned}
 1 - OR^\Lambda(t) &= 1 - \left( \frac{\alpha_{VI} \alpha_{UN}}{\alpha_{UI} \alpha_{VN}} \right) \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) \\
 &\quad \times [(1 - \phi)e^{-\lambda_{I,t}(\alpha_{VI} - \alpha_{UI})} + \phi\theta e^{-\lambda_{I,t}(\alpha_{VI}\theta - \alpha_{UI})}]. \tag{4b}
 \end{aligned}$$



These circumstances underscore that differential vaccine uptake among persons at high and low risk for infection or for symptoms given infection—a well-known phenomenon in observational studies of vaccines and other health interventions—could undermine causal interpretations of the odds ratio in test-negative studies.

**BIAS ASSOCIATED WITH DIFFERENTIAL TREATMENT SEEKING AMONG THE VACCINATED AND UNVACCINATED**

To this point we have considered ARI as a singular clinical entity and assumed all individuals seeking care for ARI are tested for influenza. However, different infections can cause clinically distinct presentations, influencing the likelihood that individuals seek treatment or the likelihood that clinicians test for influenza (46). Here we address the possibility for such a scenario to lead to selection bias from conditioning on the collider *T* (testing), the pathway  $V \leftarrow H \rightarrow T \leftarrow I$  in Figure 3.

Consider that the spectrum of clinical presentations can be discretized into “moderate” (*M*) and “severe” (*S*) classes, occurring with probabilities  $\pi_{VI} = \pi_{VI}^M + \pi_{VI}^S$ ,  $\pi_{UI} = \pi_{UI}^M + \pi_{UI}^S$ ,  $\pi_{VN} = \pi_{VN}^M + \pi_{VN}^S$ , and  $\pi_{UN} = \pi_{UN}^M + \pi_{UN}^S$ . We defined  $\mu_V^M$ ,  $\mu_V^S$ ,  $\mu_U^M$ , and  $\mu_U^S$  as the associated probabilities of seeking care given symptoms and vaccination status, and let  $\xi^M$  and  $\xi^S$  indicate the probabilities of receiving a test given symptoms. Bias associated with differential treatment-seeking persists unless the relative risk of testing given infection (which includes experiencing symptoms, seeking treatment, and being tested) does not differ for influenza and other conditions:

$$\frac{\pi_{VI}^M \mu_V^M \xi^M + \pi_{VI}^S \mu_V^S \xi^S}{\pi_{VN}^M \mu_V^M \xi^M + \pi_{VN}^S \mu_V^S \xi^S} = \frac{\pi_{UI}^M \mu_U^M \xi^M + \pi_{UI}^S \mu_U^S \xi^S}{\pi_{UN}^M \mu_U^M \xi^M + \pi_{UN}^S \mu_U^S \xi^S};$$

we derive the associated VE estimators in Web Appendix 3. Expressed more generally, this bias arises unless

$$\frac{\Pr(\text{Test}|V, I)}{\Pr(\text{Test}|U, I)} = \frac{\Pr(\text{Test}|V, N)}{\Pr(\text{Test}|U, N)} \tag{5}$$

when accommodating all possible factors that influence whether individuals are tested. Ensuring that the above condition is met can guide study implementation and circumvent possible biases owing to associations of vaccination with care-seeking given illness, receipt of clinical testing, and willingness to participate in the study.

A possible correction exists when enrollment and testing are tied to stringently defined clinical criteria (i.e., criteria for which equation 5 holds). For example, if tests are performed contingent on cases resembling a well-defined and monotypic “severe” entity (substituting  $\xi^M = 0$  in equations 5a and 5b), the odds ratio retains bias only from differential infection rates and symptom risk between the vaccinated and unvaccinated:

$$\begin{aligned} 1 - OR^C(t) &= 1 - (1 - \varphi) \left( \frac{\alpha_{UN}}{\alpha_{VN}} \right) \left( \frac{\pi_{VI}^S \pi_{UN}^S}{\pi_{UI}^S \pi_{VN}^S} \right) \\ &\times \left( \frac{(1 - \varphi)(1 - e^{-\alpha_{VI}\lambda_{VI}t}) + \varphi(1 - e^{-\alpha_{VI}\theta\lambda_{VI}t})}{1 - e^{-\alpha_{VI}\lambda_{VI}t}} \right) \end{aligned} \tag{6a}$$

when measured from cumulative incidence or

$$\begin{aligned} 1 - OR^\Lambda(t) &= 1 - (1 - \varphi) \left( \frac{\alpha_{VI} \alpha_{UN}}{\alpha_{UI} \alpha_{VN}} \right) \left( \frac{\pi_{VI}^S \pi_{UN}^S}{\pi_{UI}^S \pi_{VN}^S} \right) \\ &\times [(1 - \varphi)e^{-\lambda_{VI}t(\alpha_{VI} - \alpha_{UI})} + \varphi\theta e^{-\lambda_{VI}t(\alpha_{VI}\theta - \alpha_{UI})}] \end{aligned} \tag{6b}$$

when measured from the ascertainment rate (resembling equations 4a and 4b). Absent any association of the decision to receive the vaccine with individuals’ exposure or susceptibility to infection and ARI, equations 6a and 6b reduce to equations 1a and 1b.

**MEASURING VACCINE EFFECTIVENESS AGAINST PROGRESSION**

In addition to protection against infection, reductions in symptom risk given infection are of interest in VE measures (17). Let  $\rho$  be defined as the relative risk for vaccine-protected individuals to experience symptoms given infection owing to vaccine-derived immunity. When decisions to vaccinate are not correlated with exposure or susceptibility to the infections, other than through vaccine-derived immunity,

$$\Lambda_{VI}(t) = \lambda_{VI} \pi_I \mu_V [(1 - \varphi)e^{-\lambda_{VI}t} + \varphi\rho\theta e^{-\theta\lambda_{VI}t}] vP$$

and

$$C_{VI}(t) = \pi_I \mu_V [(1 - \varphi)(1 - e^{-\lambda_{VI}t}) + \varphi\rho(1 - e^{-\theta\lambda_{VI}t})] vP,$$

so that

$$\begin{aligned} 1 - OR^C(t) &= \varphi \left( 1 - \frac{\rho(1 - e^{-\theta\lambda_{VI}t})}{1 - e^{-\lambda_{VI}t}} \right) \\ 1 - OR^\Lambda(t) &= \varphi(1 - \rho\theta e^{-\lambda_{VI}t(\theta-1)}). \end{aligned} \tag{7}$$

Under the special case that a vaccine reduces risk of symptoms without protecting against infection ( $\theta = 1$ )—as might apply to oral cholera vaccines (47–49)—these measures reduce to

$$1 - OR^C(t) = 1 - OR^\Lambda = \varphi(1 - \rho),$$

an unbiased estimate of VE against progression. Under confounding between vaccination and exposure or susceptibility to the infections,

$$\begin{aligned}
& 1 - OR^C(t) \\
&= 1 - \frac{\alpha_{UN}}{\alpha_{VN}} \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) \\
&\quad \times \left( \frac{(1 - \varphi)(1 - e^{-\alpha_{VI}\lambda t}) + \varphi\rho(1 - e^{-\alpha_{VI}\theta\lambda t})}{1 - e^{-\alpha_{UI}\lambda t}} \right) \\
& 1 - OR^\Lambda(t) \\
&= 1 - \left( \frac{\alpha_{VI} \alpha_{UN}}{\alpha_{UI} \alpha_{VN}} \right) \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) [(1 - \varphi)e^{-\lambda t(\alpha_{VI} - \alpha_{UI})} \\
&\quad + \varphi\rho\theta e^{-\lambda t(\alpha_{VI}\theta - \alpha_{UI})}], \tag{8}
\end{aligned}$$

reducing to

$$\begin{aligned}
1 - OR^C(t) &= 1 - \frac{\alpha_{UN}}{\alpha_{VN}} \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) \\
&\quad \times \left( \frac{1 - e^{-\alpha_{VI}\lambda t}}{1 - e^{-\alpha_{UI}\lambda t}} \right) [1 - \varphi(1 - \rho)] \\
1 - OR^\Lambda(t) &= 1 - \left( \frac{\alpha_{VI} \alpha_{UN}}{\alpha_{UI} \alpha_{VN}} \right) \left( \frac{\pi_{VI} \pi_{UN}}{\pi_{UI} \pi_{VN}} \right) \\
&\quad \times [1 - \varphi(1 - \rho)] e^{-\lambda t(\alpha_{VI} - \alpha_{UI})}
\end{aligned}$$

for a vaccine protecting against symptoms only ( $\theta = 1$ ).

## IMPLICATIONS

Recent years have seen growing enthusiasm for the integration of data from observational studies in decisions surrounding influenza vaccine policy (50), in part based on a belief that vaccine direct effects—which have traditionally been measured in prospective, randomized controlled trials—can be recovered under the test-negative design (6, 10, 16, 24). However, uptake of the test-negative design by researchers and policy makers has preceded thorough examination of its theoretical justification (14). Our analysis highlights limitations to interpreting VE estimates based on the exposure odds ratio from test-negative studies.

Our most troubling finding is that the odds ratio measured by test-negative studies is unsuited to estimating the vaccine direct effect on susceptibility to infection even under circumstances consistent with randomized vaccine allocation, unless protection is known to follow an “all-or-nothing” mechanism of action. These results echo longstanding concerns about measurement of the effectiveness of “leaky” vaccines in case-control studies (23, 51–53) as well as clinical trials (36, 37). The underlying bias occurs because unvaccinated persons become immune via natural infection faster than vaccinated ones, causing the groups to appear more similar over time. Researchers rarely know a priori to what extent a vaccine confers “leaky” or “all-or-nothing” protection, making it difficult to know under what circumstances studies might be subject to the resulting bias.

We also showed that certain traditionally recognized sources of confounding in observational studies—arising due to differential exposure or susceptibility to infection and symptoms among vaccinated and unvaccinated persons—persist under the

test-negative design. Because resulting biases could lead to time-varying estimates of VE, declines in  $1 -$  odds ratio over a season might not support inference of waning vaccine protection (31–35). Last, whereas the test-negative design has been viewed as a strategy to eliminate treatment-seeking bias, we found that that bias could persist under differential symptom severity for influenza and test-negative infections.

Several assessments of test-negative studies based on directed acyclic graphs (2, 11) have pointed to similar sources of confounding, and the practical importance of these findings has been debated amid uncertainty about the magnitude of associated bias in estimates (16). The framework we have used provides a basis for quantifying bias directly. We showed that the odds ratio of test-negative studies can supply VE estimates that are not equal to the causal vaccine effect on susceptibility and that sign bias could arise such that the instantaneous odds ratio leads to incorrect inferences about whether a vaccine is effective or not. This is contrary to the frequent assumption that the odds ratio provides, at minimum, a valid and direction-unbiased test of the null hypothesis of no causal effect (11).

Other approaches have been taken to assess bias in test-negative studies. In informal comparisons, VE estimates from test-negative studies of live oral rotavirus vaccines and oral cholera vaccines have appeared similar to VE estimates from randomized controlled trials in the same settings (54, 55). While these findings could suggest that the biases we identify are not always large in practice, our study and others (36, 37) have pointed to potential sources of bias that could also affect estimates of the vaccine direct effect in randomized controlled trials. Moreover, seasonal influenza vaccine trials are not conducted on a year-to-year basis amid alterations to the strain composition of vaccines and changes to the immune profile of hosts. This has led to difficulty accounting for instances where conclusions of randomized controlled trials and test-negative studies have appeared to be in conflict. For instance, effectiveness of live attenuated influenza vaccine has appeared poor in test-negative studies undertaken since the emergence in 2009 of a novel H1N1 influenza A virus (56, 57), despite superior efficacy of live attenuated influenza vaccine over inactivated influenza vaccine among children in earlier randomized controlled trials (58–60).

Many of the biases we identified result from differential acquisition of natural immunity among vaccinated and unvaccinated persons. The strength and duration of such immunity differs among infectious diseases for which test-negative studies have been undertaken to estimate VE; specific implications for weakly immunizing infections such as rotavirus (18, 19) and respiratory bacterial agents (4, 22) should be assessed. Uses of the test-negative design in increasingly innovative applications, such as an evaluation of cluster-randomized deployments of *Wolbachia*-infected mosquitoes to prevent dengue (61, 62), further merit consideration in terms of transmission dynamic parameters such as those we consider here.

## STRATEGIES TO COUNTERACT BIAS

While our analysis identified limitations to the validity of VE estimates based on the vaccine-exposure odds ratio under the test-negative design, the results highlight specific improvements

that can be made to the interpretation of data from test-negative studies. We have shown that the use of strict clinical criteria or case definitions for enrollment and testing can reduce bias due to differential health-care-seeking behavior among vaccinated and unvaccinated persons. Whereas test-negative studies typically stratify estimates according to influenza type/subtype or even the genetic clade, our findings suggest bias might persist if there are meaningful epidemiologic differences in risk factors for infection and disease among vaccinated and unvaccinated persons. This bias can be reduced by stratifying estimates to minimize within-stratum differences in exposure or susceptibility to infection among vaccinated and unvaccinated persons. While we point out the inability of test-negative studies to measure “leaky” or “leaky-or-nothing” protection accurately, the persistence of such bias in randomized controlled trials echoes a broader need to consider epidemiologic approaches for the measurement of imperfect forms of immunity (23, 51–53). Because biases resulting from the “leaky” nature of vaccine protection have lower impact in populations less exposed to transmission, VE estimates from early in the influenza season might be more reliable than those obtained later. This circumstance suggests a need to maximize statistical power for test-negative studies in the initial weeks or months of seasonal or pandemic influenza transmission. In addition, monitoring the cumulative incidence of infections in populations (for example, through serological studies) could facilitate correction for the differential prevalence of naturally acquired immunity among vaccinated and unvaccinated persons. Evidence from test-negative studies of VE against influenza should be interpreted with the limitations we report here in mind, in particular for vaccination policy making.

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