CAGT-3′) and the reverse primer ITS4 (ref. 23). The size of the probes were 274 bp and 258 bp for T2 and T4, respectively. Probes were labelled by polymerase chain reaction (PCR) with DIG-11-dUTP and Biotin-16-dUTP (Roche) and purified with QiaQuick PCR purification kit (Qiagen).

Spores of *S. castanea*, *G. geosporum* (BEG 18) and *Glomus* sp. (BEG 19) were collected from pot cultures and immediately fixed (in 4% formaldehyde, 100 mM Tris/HCl, pH 8, 100 mM NaCl, 2 mM $MgCl_2$ and 0.05% Triton X100, for 2 h at room temperature). Five spores or single spores were crushed on Frost Plus slides (Polylabo) and then dried overnight. For single-target FISH, 25 μl of the hybridization solution was loaded per slide. For double-target FISH, a mixture of digoxigenin and biotin probes in a 1:1 ratio was added to the hybridization solution. Hybridization was carried out at 37 °C overnight. The post-hybridization washes were made twice with 50% formamide in double-strength SSC (42 °C, 10 min), then twice with double-strength SSC, (37 °C, 5 min) and then rinsed again twice with half-strength SSC with 0.1% SDS (60 °C, 15 min). The post-hybridization washes were identical to those used for the slot-blot procedure. For full details regarding slot-blot conditions and slide preparation for FISH and all controls see the Supplementary Information.

For signal detection in single-target FISH, slides were incubated with anti-DIG-fluorescein conjugate antibody (Roche) and counterstained with propidium iodide. In double-target FISH experiments, signals were detected with a mixture of anti-DIG-fluorescein conjugate antibody (Roche) and Streptavidin Texas-Red conjugate. The slides were counterstained with TOTO-3 (molecular Probes). Slides were examined using a scanning confocal microscope (further details are given on signal detection and microscopy in the Supplementary Information).

Testing for detection of recombination

Thirty identical data sets were constructed, representing 30 spores of arbuscular mycorrhizal fungi, each containing a population of 50 genetically different nuclei that were variable at 15 loci. The nuclei within each spore were then recombined by the random rearrangement of alleles among nuclei, assuming that loci were independent of each other. The frequency of genetically different nuclei occurring in each spore was then altered by randomly selecting 10 nuclear genotypes that were then replicated a random number of times in the spore, with a maximum limit of 10 replicates of a given nuclear genotype per spore. The number of nuclei showing the presence of an allele at each locus was summed for each spore. The presence or absence of an allele at each locus was then calculated for each whole spore using different sensitivities of the fingerprinting method to give binary data sets. Sensitivity was defined by the percentage of nuclei containing a given allele that was required to give a positive signal with a fingerprinting technique. The sensitivities ranged from 100% (where the presence of an allele in 1 nucleus in a spore was sufficient to give a positive signal with a fingerprinting technique) through to 0% (where even if every nucleus contained the allele the technique would not be sensitive enough to detect presence of the allele). Polymorphism among the 30 spores at the 15 loci was calculated for data sets at 20%, 30%, 40%, 50%, 60%, 70% and 80% sensitivities. Sensitivity was calculated on a percentage basis rather than actual numbers of nuclei to account for differences in the numbers of nuclei among spores. Each of these seven data sets were then separately used to calculate the index of association[16].

Incompatibility analysis on rDNA and *BiP* gene sequences

Incompatibility analysis was performed on sequences of ITS and 28S rDNA from different arbuscular mycorrhizal fungi species. ITS sequences of *G. geosporum* (BEG 18) were obtained by extracting genomic DNA from spores (Qiagen DNeasy Plant Mini Kit) from a culture that originated from a single spore and amplified by PCR[9]. The 640-bp product containing ITS1, the 5.8S gene and ITS2 was purified (Qiagen QiaQuick purification kit), ligated into a pGEM-T vector and transformed into *Escherichia coli* JM109 (Promega). Clones were selected at random and both strands were sequenced using a dye-terminator cycle sequencing kit. Sequences were carefully edited by hand. ITS sequences from *G. geosporum* (BEG 18) and from *G. mosseae*[24] and *G. margarita*[24] were aligned. Sequences of the 28S gene for the species *G. geosporum* (BEG 11)[18], *G. coronatum* (BEG 49)[18], *G. constrictum* (BEG 130)[18] and *G. mosseae* (BEG 25)[18] were also aligned and used for the analysis. All isolates originated from single spores except *G. coronatum* and *G. constrictum*. Further details on the compatibility analysis on rDNA and the gene encoding a binding protein are given in the Supplementary Information.

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Transmission potential of smallpox in contemporary populations

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Despite eradication[1], smallpox still presents a risk to public health whilst laboratory stocks of virus remain[2,3]. One factor crucial to any assessment of this risk is R_0, the average number of secondary cases infected by each primary case. However, recently applied estimates have varied too widely (R_0 from 1.5 to >20) to be of practical use, and often appear to disregard contingent factors such as socio-economic conditions and herd immunity[4–8]. Here we use epidemic modelling[9] to show a more consistent derivation of R_0. In isolated pre-twentieth century populations[10–12] with negligible herd immunity, the numbers of cases initially rose exponentially, with an R_0 between 3.5 and 6. Before outbreak controls were applied, smallpox also demonstrated similar levels of transmission in 30 sporadic outbreaks in twentieth century Europe[1], taking into account pre-existing vaccination levels[13,14] (about 50%) and the role of hospitals in doubling early transmission. Should smallpox recur, such estimates of transmission potential (R_0

from 3.5 to 6) predict a reasonably rapid epidemic rise before the implementation of public health interventions, because little residual herd immunity exists now that vaccination has ceased.

Before eradication, smallpox caused significant epidemics and mortality[1]. Following the WHO (World Health Organization) campaign, smallpox was eradicated by 1979 and routine vaccination gradually cased worldwide[1]. Official stocks of virus now remain in only two laboratories, one at CDC in Atlanta, Georgia, and the other at VECTOR, Novosibirsk, Russia[1]. There is concern, however, that the virus might be held clandestinely and less securely elsewhere[3]. Release of smallpox back into the population could constitute a significant public health problem on account of the transmissibility of the virus and waning immunity. The greater the transmissibility of the virus the greater its potential to spread, and the more intense are the intervention strategies needed to bring it under control. Significant discrepancies exist in the recent literature[4–8], so it is essential to provide a rational assessment of the transmissibility of smallpox by reference to adequate and relevant data.

Reports of outbreaks of smallpox caused by variola major were sought that contained sufficient numerical detail to derive estimates of transmissibility, R_0 (see Methods). Such reports are scarce, and those in Table 1 represent all that were found from the extensive published data. Data for four of the outbreaks were from the pre-vaccination era. Analysis of the cumulative smallpox deaths for Boston, USA[10] in 1721 and Burford, England[11] in 1758 (Fig. 1a, b and Table 1) gave R_0 values of 4.3 and 3.4, respectively, which produced extremely good fits to the data. As was often the case for small isolated communities, nearly the whole population of Burford was susceptible. Similarly, outbreaks in Chester[10] (1774) and Warrington[10] (1773) (Fig. 1c, d, and Table 1) gave estimates for R_0 of 5.8 and 4.7 (range for Warrington 4–5.3), respectively. In both of these outbreaks, only children less than 10 years old were afflicted, so the numbers of susceptible individuals were estimated from the relevant birth and age-dependent mortality rates (Table 1). The predicted epidemic curve fitted the data for Chester very well. For Warrington, however, a range of R_0 values was derived that depended on the estimated population size, which was calculated to be between 6,000 and 8,000, respectively. The fitted epidemic curve that is shown for Warrington, which is based on an R_0 of 4.7 and a population estimate of 7,000, followed the initial course of the observed epidemic well but over-predicted the cumulative number of deaths by the end of the epidemic. It is possible that the case fatality rate of 25% given by Creighton[10] was an overestimate as the other contemporaneous epidemics modelled here had case fatality rates of about 15%. Adjusting the case fatality rate to 15% greatly improved the fit to the data, closely fitting all 12 data points and producing an estimated R_0 value of 4.8 (see Fig. 1d and Table 1).

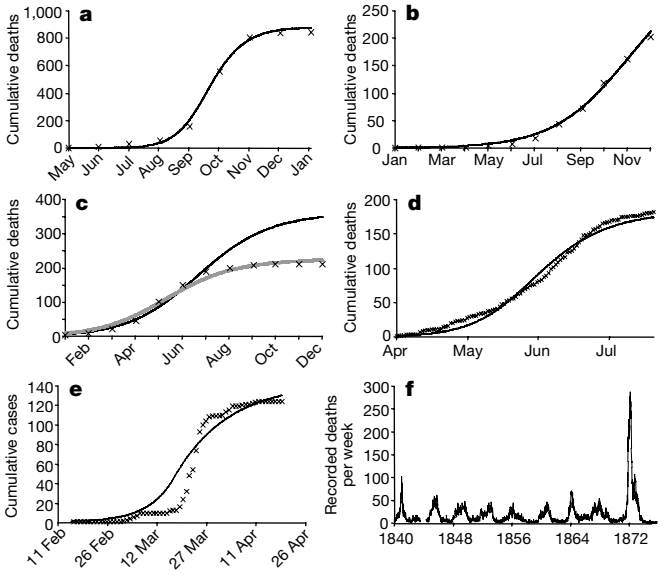

Figure 1 Analysis of smallpox epidemics. **a**–**e**, Epidemic curves (solid lines) fitted to data (points) from selected smallpox outbreaks. **a**, Boston monthly deaths; **b**, Chester monthly deaths; **c**, Warrington monthly deaths, predicted using published mortality rate of 25% (black line), or 15% (grey line; see text); **d**, Burford daily deaths; and **e**, Kosovo daily onset of cases. Disease and population parameters given in the text and Tables 1 and 2, respectively. **f**, Endemic smallpox in London with epidemics occurring approximately every 4 years[12], giving a lower bound for the estimate of R_0 of about 5; see text and Methods.

Smallpox was endemic in London from the seventeenth to the twentieth century. Weekly mortality statistics were recorded, first in the London Bills of Mortality and later in the Registrar General's reports[12]. Superimposed on an endemic level of disease, epidemics generated a cyclical pattern of smallpox deaths[15] (Fig. 1f). Such patterns are characteristic of the dynamics of endemic diseases and the models that are often used to describe them[9]. Analysis of these individual epidemics using the methods described above was not possible because independent estimates of the numbers susceptible in the population could not be made. The poorly documented levels of variolation[1] and vaccination that were being practised compounded the problem. If the problems due to vaccination are neglected, the inter-epidemic interval[15] between 1836 and 1890

Table 1 Population parameters and analysis of smallpox epidemics for estimation of R_0

Outbreak	Population size	Number susceptible	Case fatality rate (%)	Estimated R_0	Reference
Boston, USA (1721)	10,565*	6,739*	14*	4.3	10
Burford (1758)	1,520*	1,519*	13*	3.4	11
Chester (1774)	14,713*	3,167†	15*	5.8	10
Warrington (1773)	7,000 (6,000–8,000)§	2,250‡	25*	4.7 (4.0–5.3)	10
Paris, France (1766)	NA	NA	NA	>4–5¶	16
London (1836–1870)	NA	NA	NA	>5¶	12,10,15
Kosovo (1972)	2,200,000#	1,100,000☆	10**	10.8☆	1
				5.4 (discounting hospital-associated cases)††	
Europe (1958–1973)	NA	NA	10**	10 to 12	1
(Summary of 30 importations)‡‡				5 to 6 (discounting hospital-associated cases)††	

* Given in source.
† Only individuals ≤10 years were susceptible, number estimated from a birth rate of 0.03 (England) and age-dependent mortality rates (Chester).
‡ Only individuals ≤10 years were susceptible, number estimated from birth rate 0.025 and age-dependent mortality rates (Warrington).
§ Range estimated from population size of 9,501 given for 1781 adjusted for births after 1773 (Warrington's population was expanding due to industrial success).
‖ A revised estimate for case fatality rate (see text).
¶ London estimated from interepidemic interval (R not R_0 because of vaccination) and Paris estimated from force of infection and life expectancy (see text).
# 1998 estimate (http://archive.tol.cz/countries/yugar98.html), value not critical as epidemic was quickly stopped with only 125 cases.
☆ Estimate based on 50% vaccination coverage (see text).
** Given in source but not critical as model was fitted to cases not deaths.
†† Hospital-associated cases accounted for 50% of transmission.
‡‡ Estimate of R_0 based on the increasing number of cases per importation caused by increasing delays in outbreak notification (see text).
NA, not applicable.

Table 2 Interventions imposed for smallpox outbreak in Kosovo, 1972

Parameter	Value	Meaning	Reference
χ_1	0.06 days^{-1}	(Contact quarantine period)$^{-1}$	24
χ_2	0.04 days^{-1}	(Infectious quarantine period)$^{-1}$	1
ϵ_1	0.975	Vaccine efficacy when uninfected	13, 23
ϵ_2	0.30	Vaccine efficacy when infected and latent	1, 23
ρ	0.975	Proportion of contacts found	1
θ	0.95	Daily removal rate of infectious individuals into quarantine	1

suggests that the transmission rate, R (see Methods) was about 5. However, vaccination was being practised, so this provides a lower bound for the estimate of R_0. Interepidemic intervals as low as 2–3 years[15] and presumably higher values of R_0 had also applied previously in London, and these have been variously correlated with greater susceptibility to disease, greater density of population or lack of vaccination. Dietz and Heesterbeek[16] also report on an analysis of smallpox in Paris by Bernoulli in 1766. His estimated force of infection, λ, of 0.125, and life expectancy, L, of 32 years, give an estimated R_0 of between 4 and 5 for smallpox in this other major European city (given that[9] $R_0 = \lambda L$ or $R_0 = 1 + \lambda L$).

Although no longer endemic in Europe by the early 1950s, smallpox still caused isolated epidemics after the importation of the disease from endemic areas despite the considerable level of herd immunity in European populations[1,13,17]. One of the best documented epidemics was in Yugoslavia in 1972 (ref. 1). Starting in Kosovo, the outbreak went unrecognized until the second generation of infection, when vaccination and quarantine of contacts was initiated, followed by vaccination of about 95% of the population. We fitted equation (1) to the data, parameterized to take into account these interventions (Table 2), and hence estimated R (see Methods) to be about 5.4; this value fell rapidly to below 1 with the introduction of quarantine and vaccination (Fig. 1e, Table 1). However, about 50% of the population were probably protected by vaccinations that pre-dated the outbreak[13,14], such that the initial R_0 would have been nearer 10.8. The epidemic curve from equation (1) provided a reasonable overall fit to the data from Kosovo but underestimated the number of cases in the earlier part of the outbreak, probably on account of the small size of the outbreak and the fact that there were only two generations of infection before interventions were applied.

Thirty European smallpox importations between 1958 and 1973 were also briefly summarized in ref. 1. Stratified by the time delay between importation and recognition of the outbreak, and its notification to the WHO (0–7, 8–14, 15–21 and 22–42 days), the outbreaks that took longer to be notified resulted in a greater average number of cases before the epidemics were halted (5.4, 8.8, 15.8, 67.8 cases, respectively). With the median delay adjusted by the addition of a latent period for the index case and expressed in units of intergeneration time (19 days), a clear relationship was derived between the delay and the total number of cases (Fig. 2a). Each generation's delay increased the average number of cases by $e^{1.71}$, giving a mean estimate for R of 5.5 ($\mu \pm$ s.e.m. = 4.9 to 6.3). As before, these estimates are affected by previous vaccination levels; 50% vaccination, as has been suggested[13,14], would give an estimate for R_0 of about 11. Interestingly, about 50% of cases in most of these European outbreaks, including in Kosovo, were acquired in hospital by staff and patients[1] before the outbreak was recognized and hospital infection-control measures had been put in place (Fig. 2b). Without hospital-associated cases, the R_0 for smallpox would have been about 5.5 for community-acquired disease.

As expected from data on other infectious agents[9], the estimates of R_0 derived for smallpox varied with time and place. Over the 1700s to 1900s, they tended to be in the range 3.5–6, but under particular circumstances rose to nearer 10–12 (that is, with crowding and poorer socio-economic conditions in eighteenth century London[15] or when exacerbated by hospital-associated infections in more recent European outbreaks[1]). This analysis is in accordance with inferences from the vaccination level required for smallpox eradication[1,9] in industrialized countries. The value of 70–80% that was found to be effective would suggest an R_0 between 3.3 and 5 (ref. 9). However, vaccination levels of 80% were not effective in eradicating smallpox in India because of much higher population densities and poorer socio-economic conditions[18], which would imply R_0 values higher than 5. For contemporary outbreaks in industrialized countries, which now have low levels of herd immunity (18%, see Methods), an R_0 value of 4–6 would probably apply for community-acquired infections, but might transiently be higher (for example, 10–12) before the disease was correctly recognized and appropriate hospital infection controls implemented. These values differ from recently applied values. The higher values[4–6] from 10 to 20 have been estimated from atypical outbreaks with unusually high transmission rates[1,19], whereas the lower estimates of around 2 appear to have neglected concurrent herd immunity[8]. Although our estimate for smallpox represents a relatively modest transmission rate by comparison with some other infectious diseases, such as measles or chickenpox[9], significant epidemics could result, particularly if there were delays in detecting the first cases or in setting up effective public health interventions. □

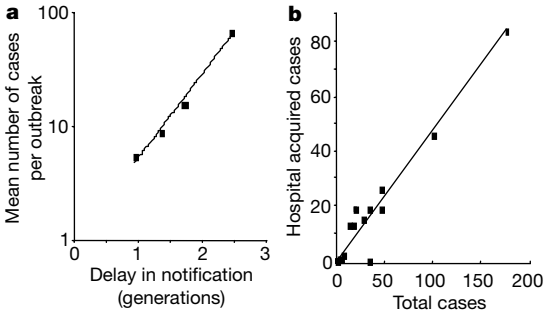

Figure 2 Analysis of 32 European smallpox outbreaks post-1950. **a**, Regression analysis demonstrating the increase in the mean number of cases per outbreak that occurred (note logarithmic scale of ordinate and abscissa) with increased median delay in notification of the outbreak (delay expressed in units of generation time of the disease (19 days)). Regression: $y = 0.95e^{1.71x}$, $r^2 = 0.99$. **b**, Regression analysis showing the relationship between the total number of cases per outbreak and the number of cases that were hospital-acquired. Regression: $y = 0.4777x$, $r^2 = 0.94$).

Methods

Epidemic modelling

For epidemic modelling, transmissibility is often expressed as the basic reproductive number, R_0, the mean number of secondary cases caused by each primary case in a population composed entirely of susceptible individuals[9]. The reproductive number, R, is an equivalent measure, but without reference to the proportion of susceptible hosts[9]. Smallpox infection results first in a non-infectious incubation period followed by a short prodrome with non-specific symptoms (assumed to be non-infectious[20]), which together constitute the latency period. Hosts then become overtly infectious and symptomatic, with a pustular rash and, thereafter, slowly recover and are immune, or die[1,17]. Whilst infectious, hosts transmit disease to susceptible individuals at a rate dependent on R_0. Public health interventions interrupt transmission by removing infectious individuals into quarantine, or by reducing the susceptibility of contacts by vaccination[9]. These processes were modelled using an appropriately parameterized set of differential equations (1), which were solved using Euler's method with a time-step of 0.1 days.

$$\begin{aligned}
\frac{dS}{dt} &= \chi_1(1-\epsilon_1)C_i - \beta(\varphi+\rho-\varphi\rho)SI \qquad \frac{dI}{dt} = \alpha(1-\theta)E_n - (\theta+\gamma)I \\
\frac{dE_n}{dt} &= \beta\varphi(1-\rho)SI - \alpha E_n \qquad \frac{dQ}{dt} = \alpha(1-\epsilon_2)E_i + \theta(\alpha E_n + I) - \chi_2 Q \\
\frac{dE_i}{dt} &= \beta\varphi\rho SI - (\chi_1\epsilon_2 + \alpha(1-\epsilon_2))E_i \qquad \frac{dU}{dt} = \gamma I + \chi_2 Q \\
\frac{dC_i}{dt} &= \beta\rho(1-\varphi)SI - \chi_1 C_i \qquad \frac{dV}{dt} = \chi_1(\epsilon_2 E_i + \epsilon_1 C_i)
\end{aligned} \tag{1}$$

S and *I* represent the proportion of the population susceptible and infectious, respectively. *U* represents the proportion dead and recovered, with the number dead dependent on the specific case fatality rates given in the data sets. The contacts of cases are divided into the following classes: E_n the number of untraced latent individuals in the population, E_i the number of traced latent contacts, and C_i the number of traced uninfected contacts. The final class of contacts are those untraced and uninfected and so effectively remaining in *S*. *Q* represents the proportion in quarantine and *V* the proportion protected by vaccination. The average rate at which latent individuals become infectious[13,21] is $\alpha = (\text{latency period})^{-1} = 0.0685\ \text{days}^{-1}$ and the rate at which infectious individuals in the community recover or die[22] is $\gamma = (\text{infectious period})^{-1} = 0.116\ \text{days}^{-1}$. Two states of quarantine are defined: the first for the traced contacts successfully vaccinated and released into the community at a rate χ_1, and the second for the infectious cases, which enter *U* at a rate χ_2. Different vaccine efficacies are assumed for those uninfected, ϵ_1, and infected, ϵ_2. The proportion of contacts found through contact tracing is ρ and the daily rate at which infectious individuals enter quarantine from the community is θ. The proportion of contacts infected is defined as φ. The rate at which potentially infected contacts occur is defined as β, as in equation (2), and *N* is the size of the population in which the epidemic occurs.

$$\beta = \frac{R_0\gamma}{\varphi N} \tag{2}$$

Additional assumptions are that no transmission occurs from those quarantined, dead or recovered and the background mortality rate was assumed to be negligible over the time periods examined.

For the Boston, Burford, Warrington and Chester data sets, $\rho = \theta = 0$, which effectively reduces equations (1) above to a simple SEIR model[9]. Intervention parameters were only required when equations (1) was fitted to the data from Kosovo. Here, interventions were implemented 31 days after the onset of symptoms in the index case[1] with the associated parameters shown in Table 2. The number of potentially infected contacts per case was determined as 50 (ref. 1). Values of R_0 were derived for each outbreak by minimizing the mean square error between the mortality data and the predictions of mortality from the model, while applying the outbreak-specific case fatality rates to *U* and adjusting R_0 and time of onset of symptoms in the index case. In the case of Kosovo, equations (1) were fitted more simply to the reported number of cases rather than deaths. All the other parameters required for equations (1) were obtained independently from the published source(s) given in Table 1. For epidemics in London, R_0 was roughly calculated from the interepidemic interval, $T = 2\pi[L(D + D')/(R_0 - 1)]^{1/2}$, where *L* is life expectancy between 1840 and 1870 adjusted for excess births over deaths, equal to 25 years, and $D + D'$ is latent + infectious period, equal to 0.063 years[9].

Estimation of current vaccination coverage

Given that smallpox vaccination ceased in industrialized countries in the mid to late 1970s (ref. 13), a crude estimate of the immunity of the contemporary UK population was calculated, on the basis of 50% having been vaccinated as infants up to 1972, and estimating that about 60% of these would be alive today from current population statistics. Of these only about 60% would still be protected by the vaccinations done on average 50 years previously, calculated by extrapolating from data on secondary attack rates, which increased from 4 to 12% over 10 years following vaccination[23]. This suggests that the level of herd immunity may be about 18%, which will continue to decrease with time.

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Imperfect vaccines and the evolution of pathogen virulence

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Vaccines rarely provide full protection from disease. Nevertheless, partially effective (imperfect) vaccines may be used to protect both individuals and whole populations[1–3]. We studied the potential impact of different types of imperfect vaccines on the evolution of pathogen virulence (induced host mortality) and the consequences for public health. Here we show that vaccines designed to reduce pathogen growth rate and/or toxicity diminish selection against virulent pathogens. The subsequent evolution leads to higher levels of intrinsic virulence and hence to more severe disease in unvaccinated individuals. This evolution can erode any population-wide benefits such that overall mortality rates are unaffected, or even increase, with the level of vaccination coverage. In contrast, infection-blocking vaccines induce no such effects, and can even select for lower virulence. These findings have policy implications for the development and use of vaccines that are not expected to provide full immunity, such as candidate vaccines for malaria[4].

Previous studies on the evolution of vaccine resistance have focused on the spread of 'escape' mutants that display epitopes different to those in the vaccine, thereby escaping immune recognition[5–7]—this has already happened for polio[8] and hepatitis B[9]. New vaccines may eventually get around this problem by, for example, targeting conserved epitopes or multiple epitopes simultaneously. Here we study an alternative counter-adaptation to vaccination involving pathogen life-history traits, namely virulence (induced host mortality) and transmission rate. To address this issue we incorporated standard evolutionary theory for virulence evolution[10–12] into an epidemiological framework[1].

We begin with an analysis of the evolution of parasite virulence

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correction

Transmission potential of smallpox in contemporary populations

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There was an error in equations (1) in this Letter. The θI terms in dI/dt and dQ/dt should have been omitted. Thus, the set of differential equations should have been:

$$\begin{aligned}
\frac{dS}{dt} &= \chi_1(1-\epsilon_1)C_i - \beta(\varphi+\rho-\varphi\rho)SI & \frac{dI}{dt} &= \alpha(1-\theta)E_n - \gamma I \\
\frac{dE_n}{dt} &= \beta\varphi(1-\rho)SI - \alpha E_n & \frac{dQ}{dt} &= \alpha(1-\epsilon_2)E_i + \alpha\theta E_n - \chi_2 Q \\
\frac{dE_i}{dt} &= \beta\varphi\rho SI - (\chi_1\epsilon_2 + \alpha(1-\epsilon_2))E_i & \frac{dU}{dt} &= \gamma I + \chi_2 Q \\
\frac{dC_i}{dt} &= \beta\rho(1-\varphi)SI - \chi_1 C_i & \frac{dV}{dt} &= \chi_1(\epsilon_2 E_i + \epsilon_1 C_i)
\end{aligned} \qquad (1)$$

and the definition of θ in Table 2 should have read 'the proportion of infectious individuals from E_n that are quarantined'. This change only affects the analysis of the data for Kosovo. The value of R_0 that gives the best fit to the data for Kosovo is now 10 (5 discounting hospital-associated cases), rather than 10.8 (5.4 discounting hospital-?associated cases), as stated in the original paper in Table 1. This small error does not alter our conclusions. □