Prevalence and implications of multiple-strain infections

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Infections frequently contain multiple strains (genotypes) of the same pathogen, yet they are still usually treated as uniform entities. In this Review, we discuss problems with inconsistent definition of the term "strain" and review the prevalence and implications of multiple-strain infections. Up to now, multiple-strain infections have been shown unambiguously in 51 human pathogens (and 21 non-human ones) and are likely to arise in most pathogen species. In human pathogens, multiple-strain infections usually reach considerable frequencies (median 11·3%, mean 21·7% of infections), which are certainly underestimated in many cases because of technical limitations of detection. For many diseases, the importance of multiple-strain infections is still unclear, but theoretical work and experimental results from animal models suggest a broad range of clinically relevant effects. Multiple-strain infections can affect host immune responses and our ability to prevent and treat infection efficiently. Competition and mutualism between strains change pathogen and disease dynamics and promote pathogen evolution. Co-infection enables gene transfer among strains. Taking multiple-strain infections into account will improve our understanding of host-pathogen interactions and disease dynamics, and will provide a basis for novel control approaches.

Introduction

Pathogens are still sometimes regarded as uniform entities despite growing awareness that infections frequently, if not usually, consist of more than one pathogen species or multiple strains of the same species. Much work on infections with multiple pathogen species has been published.[1] However, apart from a few prominent cases, in most diseases, multiple-strain infections are just beginning to be considered. The consequences of multiple-strain infections for evolution of pathogens and expression of disease have attracted great theoretical interest.[2–4] However, empirical investigations of the frequency and effects of multiple-strain infections on disease dynamics, pathogenicity, drug treatment, and vaccination are still limited because of technical challenges of efficiently distinguishing individual strains of many disease agents.

In this Review, we aim to show how prevalent multiple-strain infections are in disease-causing protozoa, helminths, bacteria, fungi, and viruses and what implications these infections might have. Since data are still limited from human pathogen systems, studies of non-human pathogens will be discussed when they provide insights relevant to human disease. Our focus is on empirical studies but theoretical or modelling studies are included when they illustrate ideas particularly well.

Definitions and approach

Great heterogeneity exists across areas of research with respect to use of the terms "strain", "genotype", "clone", and "isolate". In this Review, we use the term strain without implying that our definition is more correct than others. As a general definition, strains are homogeneous groups within species—ie, they are more similar within than between groups. Definitions vary with respect to traits used and the degree of homogeneity needed to delineate strains. The definition used can depend on methods available for discrimination and on the exact questions being asked in different studies. Strains can be defined by their homogeneity in non-coding DNA sequences (eg, microsatellites) or in specific genetically determined traits of interest (eg, drug resistance, virulence factors). Strains are also sometimes defined on the basis of structural features (eg, epitopes) or medical properties (eg, the symptoms they cause), which do not necessarily reflect genetic relations between strains. In practice, the term strain is sometimes used synonymously with isolate (or with cultured isolate)—ie, a line of parasites that goes back to an uncloned parasite population that was extracted from a host individual on one occasion. This definition is problematic because isolates can contain several genetic strains and because different isolates can consist of identical pathogen populations. In the strictest definition, a strain comprises all pathogens that share an identical genome sequence by descent.

Strains are important because they can differ greatly in many traits, including growth rate,[5] virulence,[6–8] infectivity,[9] antigenicity,[10] or drug resistance.[11] The classification of pathogens into strains is thus of practical value. However, because of little commonality in use of the term strain, researchers should always state what definition they used to avoid misunderstandings. In this Review, we only consider genetic strain definitions in our discussion of the implications of multiple-strain infections. In our analysis of empirical published work, we follow the definitions used by the authors.

Detection of multiple-strain infections

The likelihood of detecting multiple-strain infections varies greatly depending on the methods used to detect them. Multiple-strain infections can be detected by any standard typing strategy capable of distinguishing individual strains—eg, single nucleotide polymorphisms; microsatellite, minisatellite, or restriction fragment length polymorphism analysis; strain-specific PCR amplification; multilocus enzyme electrophoresis; multilocus sequence typing; or pyrosequencing.[12–16] Multiple-strain infections are detected either by recording more

alleles at a locus than are possible in just one strain (eg, more than two microsatellite alleles at a diploid locus) or by identifying dissimilar genotypes in different clones (populations grown up from one pathogen) from the same isolate. Indirect methods to infer the occurrence of multiple-strain infections—eg, detection of antibodies or recombinant genotypes—were not accepted as evidence for multiple-strain infections by us in this Review because they cannot distinguish sequential and concurrent infections. For many pathogens, either no typing strategy is available or only one with limited resolution exists. The prevalence of multiple-strain infections obtained by any method is an underestimate because rare strains are hard to detect and false-negatives can seldom be excluded completely. These difficulties, along with the scarcity of typing methods in many pathogens, means that multiple-strain infections are probably grossly underestimated in general. Viruses (and other pathogens with very high mutation rates) must be treated with additional caution. Many viruses have such high replication and mutation rates that apparent multiple-strain infections can result from within-host evolution, not initial infections with several strains.

Prevalence of multiple-strain infections

Unambiguous multiple-strain infections have so far been recorded for 51 human pathogens (table)[17–106] and 21 non-human animal pathogens (webappendix pp 1–3), numbers that suggest that multiple-strain infections are common. However, parasites are estimated to represent more than 50% of all living species, and close to 2 million species have been described scientifically,[107] so these numbers are in fact very low. However, available data strongly suggest that whenever researchers specifically look for multiple-strain infections with appropriate genetic methods, they find them. The absence of further empirical findings thus probably indicates a scarcity of methods or attention.

A median 11·3% (IQR 6·2–25·4; mean 21·7% [SD 22·5]) of all infections by human pathogens contain multiple strains (figure). Multiple-strain infections are, thus, not a rare occurrence that can be ignored. The prevalence values (table) are rough estimates because sample sizes were small in many studies and because rates will vary in space and time and with methods used. Importantly, most prevalence data must be underestimates for methodological reasons (eg, sensitivity of markers, poor detection of rare strains). Furthermore, pathogen species listed in the table are not a random subset of all pathogens: generally, they can be cultured in artificial media or laboratory rodents, are very well studied, need genetic methods for diagnosis, or a combination of these. We predict that multiple-strain infections arise in most pathogen species and that the list in the table will increase rapidly as powerful molecular methods are routinely used in an expanding set of pathogen species.

Implications of multiple-strain infections

For many pathogens, we do not understand what the specific implications of multiple-strain infections are. In most cases, this gap in knowledge exists because research has not been done.

Although some mechanisms for how multiple-strain infections affect infection dynamics are well-supported by data, others have not been confirmed empirically. For many questions, the relative frequencies of strains have to be tracked within a host over time,[108,109] which can be challenging. Furthermore, several mechanisms have not been investigated in human diseases because of general operational and ethical complexities of experiments with human diseases. One approach for analysis of pathogens in people is to do in-depth studies as part of clinical trials (particularly phases II and III), or closely related transmission studies, because these studies adhere to good clinical practice and good laboratory practice guidelines, follow fully standardised procedures, and, hence, are done under completely controlled conditions.

Mechanisms of action of multiple-strain infections can overlap with those of co-infections with more than one pathogen species. However, different species can elicit diverse immune responses[1] and vary much more than strains, leading to different dynamics.[110]

Immune defence

Multiple-strain infections may overwhelm hosts' immune systems by posing complex immune challenges. The increased number of antigenic epitopes in multiple-strain infections stimulates activation and proliferation of more lymphocyte lines, amplifying the demand on host resources. This rationale is intuitively appealing, but very few studies have tested it. In high-transmission *Plasmodium falciparum* areas, augmented numbers of co-infecting strains raise the odds of clinical malaria in babies younger than 1 year, but the correlation is reversed in children older than 1 year, in whom an increase in the number of co-infecting strains correlates with diminished disease risk.[111] This effect is probably due to premunition (resistance to new infections because of an existing infection with other strains). As a result, in low-transmission areas where no age-dependence is present, the number of co-infecting strains generally correlates with odds of disease.[112,113] A linear relation seems to exist between the number of co-infecting strains and disease risk, whereby levels of acquired immunity are low but the association wanes as acquired immunity builds up.[113]

In people with HIV-1, multiple-strain infection is associated with more rapid disease progression from seroconversion to clinical AIDS (2–4 years instead of 8–10 years),[97] but the underlying mechanisms of action are unknown. The most probable mechanisms are increased total pathogen load, accelerated adaptation to niches within the host due to greater genetic variability within the virus population, or improved immune escape through greater genetic diversity.

See Online for webappendix

	Prevalence of multiple-strain infections*	Empirical implications†
Protozoa		
Blastocystis sp[17]‡	2·6% (n=78) in China (Yunnan province)	..
Cryptosporidium parvum[18]	N/A	..
Giardia intestinalis[19,20]§	5% (n=40) in Saudi Arabian children[20]	..
Plasmodium falciparum[21–26]	83·0% (n=27) in Tanzania[22] 83·3% (n=24) in children 0–14 years in Senegal[25] 22·2% (n=27) in people >14 years in Senegal[25] 50–60% in The Gambia[23] 69·8% (n=172) in Kenya[24] 19·2% (n=104) in Sudan[22]	Immune defence; treatment and vaccination; competitive and mutualistic pathogen-pathogen interactions
Plasmodium malariae[27]	71% (n=38) in Malawi[27] 18·5% (n=27) in Thailand[27] Present in The Gambia (n=5)[27]	..
Plasmodium vivax[28,29]	65% (n=23) in Papua New Guinea[29] 20·5% (n=44) in Thailand[28] 5·6% (n=99) in China (Anhui, Guangxi, and Guizhou provinces)[28]	..
Toxoplasma gondii[30,31]	1·2% (n=84) of worldwide isolates[30] 22·2% (n=27) in fresh UK meat samples[31]	..
Trypanosoma brucei[32–34]	9·5% (n=126) in vertebrate hosts[34] 42·9% (n=28) in east African tsetse flies[32] 8·7% (n=23) in *T brucei* gambiense patients in Côte d'Ivoire[33]	Competitive pathogen-pathogen interactions; genetic exchange between pathogen strains
Trypanosoma cruzi[35,36]	4·9% (n=61) in Chile[35] 41% (n=51) in Bolivia[36]	..
Helminths		
Ascaris sp[37]	N/A	..
Fasciola hepatica[38]	90·9% (n=11) in ruminants from Ireland, the Netherlands, Greece, Australia[38]	..
Schistosoma haematobium[39]	N/A	..
Schistosoma mansoni[40,41]	54·1% (n=98) in snails in Brazil[41] 11·6% (n=43) in snails in Guadeloupe[41]	..
Bacteria		
Aggregatibacter actinomycetemcomitans[42]	9·3% (n=75) in Brazilian patients[42]	..
Borrelia burgdorferi[9,43]	6·1% (n=132) in patients in northeast USA[9] 50% (n=40) in ticks in northeast USA[43]	..
Burkholderia multivorans[44]	N/A	..
Burkholderia pseudomallei[45]	1·5% (n=133) in melioidosis patients in Thailand[45]	..
Burkholderia cepacia[46]¶	N/A	..
Campylobacter jejuni[47–49]	7·7% (n=52) in UK patients[49]	..
Chlamydia trachomatis[50–52]	41% (n=22) in Tanzanian children aged 1–2 years[50] 3·7% (n=27) in Tunisian samples[51]	..
Enterobacter cloacae[53]	N/A	..
Escherichia coli[54]	6·3% (n=32) in patients with diarrhoeagenic *E coli* infection[54]	Competitive pathogen-pathogen interactions
Haemophilus influenzae[55,56]	52·9% (n=38) in cultures from Michigan children[55]	Competitive pathogen-pathogen interactions
Helicobacter pylori[57–59]	24·0% (n=183) in Dutch patients[58] 13·4% (n=82) in Brazilian patients[59]	Treatment and vaccination
Mycobacterium avium[60,61]	43·8% (n=16) in hens in the Czech Republic[61]	..
Mycobacterium tuberculosis[62–64]	8·1% (n=37) in Spanish HIV-positive inmates[63] 18·8% (n=186) in South African patients[64]	Treatment and vaccination
Neisseria gonorrhoeae[65]	40% (n=20) in patients in the USA[65]	Competitive pathogen-pathogen interactions
Propionibacterium acnes[66]	N/A	..
Pseudomonas aeruginosa[67,68]	N/A	..
Salmonella enteritidis[69]‖	N/A	..
Staphylococcus aureus[70,71]	9·5% (n=148) in patients in USA[70]	..
Coagulase-negative staphylococci[72,73]	N/A	Treatment and vaccination
Streptococcus pneumoniae[55,74]	10·0% (n=38) in cultures from Michigan children[55]	Treatment and vaccination
Yersinia enterocolitica[75]	N/A	..

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	Prevalence of multiple-strain infections*	Empirical implications†
(Continued from previous page)		
Fungi		
Candida albicans[76]	16·7% (n=42) in HIV-positive patients in Côte d'Ivoire[76]	..
Cryptococcus neoformans[77,78]	N/A	..
Pneumocystis jirovecii[79–82]	10·5% (n=152) in the UK[79] 17·8% (n=191) in the USA[80] 16·1% in Spain[81]	..
Viruses		
Dengue virus[83,84]	11·1% (n=148) in Indonesia[83] 5·2% (n=119) in Mexico[83] 0·0% (n=74) in Puerto Rico[83] 18·8% (n=48) in India[84]	Competitive pathogen-pathogen interactions
Epstein-Barr virus[85–87]	11·5% (n=26) in HIV-positive patients[86]	..
Hepatitis B virus[88–90]	1·9% (n=212) among blood donors in Thailand[88] 3·4% (n=116) in Mongolia[90]	Treatment and vaccination
Hepatitis C virus[90–92]	10·0% (n=180) in US patients with end-stage renal disease[91] 4·9% (n=61) in Korean drug users[92] 6·6% (n=106) in Mongolia[90]	..
Hepatitis D virus[93]	38·9% (n=18) in Taiwan[93]	..
Hepatitis E virus[94]	6·5% (n=31) in Nepal[94]	..
Herpes simplex virus[95]	N/A	..
Human adenovirus[96]	4·3% (n=115) of lower-respiratory-tract infections in Korea[96]	..
HIV-1[97,98]	14·7% (n=34) in US and South African patients[97] 8·9% (n=45) in east African clinical samples[98]	Immune defence
Human cytomegalovirus[99,100]	25·8% (n=97) in samples from France[99] 2·6% (n=114) in Brazil[100]	..
Human herpesvirus 6[101]	N/A	..
Human papillomavirus[102,103]	8·9% (n=1998) in infections from nine countries[102] 23·7% (n=152) in infections in the Netherlands[103]	..
Influenza A virus[104]	1·9% (n=2839) in wild ducks in Canada[104]	Genetic exchange between pathogen strains
Rotavirus[105]	63·5% (n=104) in children in Guinea-Bissau[105]	..

N/A=frequency data not available or judged too unreliable because of low sample size or methods used. *Percentage of confirmed infections containing multiple strains; n=sample size (infected patients investigated). †See text (Implications of multiple-strain infections) for ways in which multiple-strain infections lead to substantial changes compared with single-strain infections. ‡It is not entirely clear if *Blastocystis* subtypes[106] represent strains or different species; the systematic position of *Blastocystis* sp is also controversial. §Synonyms include *Giardia duodenalis* and *Giardia lamblia*. ¶Sometimes called *Pseudomonas cepacia*. ||Synonym of *Salmonella enterica subsp enterica serovar Enteritidis*.

Table: **Multiple-strain infections of human pathogens in human and animal hosts and their empirical implications**

Treatment and vaccination

Strains can differ by their susceptibility to drugs or vaccines, which is especially apparent in drug-resistant strains.[11] Mixes of different strains increase the probability that infected hosts harbour strains that are refractory to treatment. In settings with regular drug administration (eg, hospitals) or in diseases for which strain-specific vaccines or drugs are applied, multiple-strain infections favour resistant strains that would otherwise be rare or inferior to others.[57,114–116] For specific pathogens in which drug resistance is a problem for treatment (eg, coagulase-negative staphylococci), one clone is genotyped to assess drug resistance, and treatment regimens are based on the resistance profile of this clone. This approach becomes problematic in the presence of multiple-strain infections because other strains that escape detection could be resistant to a different set of drugs, so inappropriate treatment regimens might be given.[72] If multiple-strain infections arise, this standard procedure must be changed to a protocol in which multiple clones are genotyped and, if infection persists, genotyping of further clones takes place.

Multiple-strain infections of *Leishmania infantum* and *Mycobacterium tuberculosis* complicate treatment. In a patient with *L infantum*, strain MON-1 was initially detected and cleared with standard drugs. However, infection persisted and, after renewed genotyping, a second strain—MON-98, which was resistant to the drug used—was discovered and cleared by a different drug. Experimental analysis of the two strains showed that, without drugs, MON-1 overgrows MON-98 because of a higher growth rate, which is why the second strain was initially not detected. Administration of drugs against MON-1 released MON-98 from competitive suppression.[114] The two strains had probably arisen in the patient concurrently but were not discovered together because sampling was insufficient to find the rarer strain. In a patient with *M tuberculosis*, a drug-susceptible strain

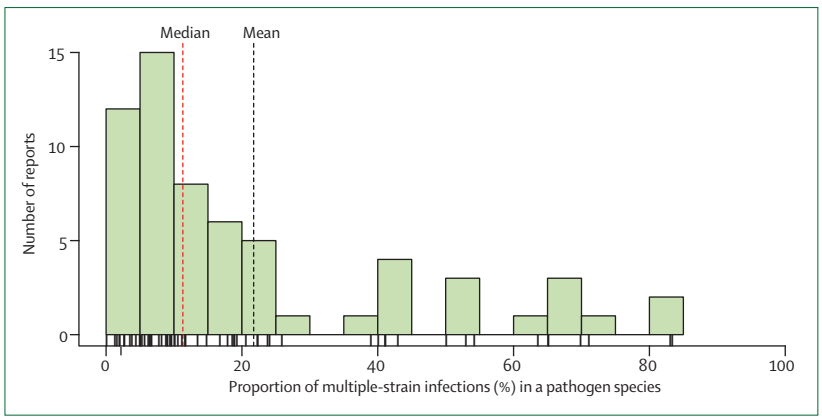

Figure: **Prevalence of multiple-strain infections in human pathogens**
Histogram of the 62 prevalence values reported in the table. Dashes along the x axis indicate individual values.

dominated before treatment with a first-line antibiotic.[117] After the first strain was cleared, another strain was detected that had to be cleared by a second-line antibiotic. Without genotyping, which confirmed the presence of distinct strains, clinical interpretation might have been conversion from susceptibility to resistance of one strain.

Selection of non-targeted genotypes by genotype-specific polyvalent vaccines has been noted in clinical trials of the malaria vaccine Combination B against *P falciparum*[118] and of five-valent and 23-valent pneumococcal vaccines.[115] In both cases, the genotypes targeted by the vaccines were significantly reduced whereas some non-targeted genotypes increased substantially. Strains of *P falciparum*, based on the genotype of merozoite surface antigen 2, cause different morbidity: FC27-like genotypes were twice as likely to cause clinical malaria than 3D7-like genotypes.[8] Experience from vaccination and vaccine trials strongly advocates for development of vaccines consisting of conserved antigens and targeting as many parasite strains as possible to minimise the chances that vaccination merely shifts relative genotype frequencies.

Competitive pathogen–pathogen interactions

Competition can take three distinct forms. First, in direct interference competition, strains excrete substances that harm each other. Excretion of toxins against competing conspecific strains is well known in *Escherichia coli* infections: strains produce highly specific toxins (known as colicins) that are active against other strains but to which they are resistant.[119] The same dynamic occurs in infections of *Haemophilus influenzae*[120] and *Streptococcus mutans*.[121] Physical aggression against other strains (eg, ingestion or membrane disruption) would have identical effects but we are not aware of evidence of this occurrence in pathogens.

Second, resource competition is an indirect form of competition in which one strain uses limited host resources that are then no longer available to the other strain. Resources can be nutrients or space—for example, pathogens such as *Plasmodium* spp compete for uninfected erythrocytes. Resource competition is an important and well documented mechanism of interaction between species and is usually invoked as the mechanism of intraspecific interaction by which pathogen population sizes are restricted to a fixed maximum attainable size or carrying capacity.[107]

Third, competition can also be mediated by the immune system through so-called apparent competition. This term was first used to describe indirect competition between unrelated prey species that are linked through a shared predator.[122] The population densities of both prey species affect the population density of predators and, thus, the amount of predation to which the other species is exposed. In immune-mediated apparent competition, the effector cells of the immune system act as the shared predator. By activating an immune response, strain A affects strain B if the response cross-reacts with strain B. This mechanism has been shown experimentally in multiple-strain infections of *Plasmodium chabaudi*.[123] The action of immune-mediated apparent competition can also be inferred from patterns of antigenicity of a pathogen. When cross-reactivity falls with increasing antigenic difference, and strains suffer from being too similar to each other, they are selected to diverge into antigenic types with little antigenic overlap.[124] This process could lead to the strain distributions noted in nature in *Neisseria meningitidis*[125] and dengue virus.[126] In *P falciparum* malaria, protection against infection by a new strain owing to the presence of other strains (premunition) is well established.[21,111] Together with semi-immunity that starts building up in early adolescence, premunition leads to striking age-dependence of the number of co-infecting strains in *P falciparum*. Age-dependence of the number of co-infecting strains could therefore be a marker to identify in which other diseases premunition and semi-immunity have an important role.

To distinguish different types of competition is not always easy. For example, *Plasmodium chabaudi* strains infected 3 days after another strain had lower growth rates.[127] This diminished growth could be due to an immune response established against the first strain. However, the first infection also depleted the number of red-blood cells, thus reducing space available for the second strain to invade. The negative effect on the second strain could thus be attributable to immune-mediated apparent competition, resource competition, or a combination.

Multiple-strain infections can also have advantageous effects for the host compared with single infections. Such benefits are clinically relevant if the changed pathogen dynamics translate into altered effects on the host, as recorded for two *Trypanosoma brucei* strains of differing virulence.[7] Hosts infected with both strains survived significantly longer than did those infected with the more virulent strain alone, despite having received the

cumulative (ie, highest) infecting dose. Tracking the population growth of both strains in single and mixed infections revealed that the two strains mutually suppressed each other. Competition between these strains benefited the host by reducing the population size of the more virulent pathogen strain, which ultimately decided the physical condition of the host. In *P chabaudi*, competition between co-infecting strains in the host can affect which strains are taken up by the vector,[6] potentially altering transmission dynamics.

Competing strains can control each other,[7] similar to the way live cells of non-pathogenic species in probiotics are used to control pathogens in gut microflora,[128] and, arguably, cases even exist whereby treatment can lead to worse outcomes for patients than non-treatment. In malaria-endemic areas, constant reinfections maintain protective immunity (premunition) against newly infecting strains.[21] Multiple infections with strains causing chronic disease thus increase protection against new and potentially more virulent infections. When semi-immune people from endemic areas go into non-malarious areas for prolonged periods they lose at least part of their semi-immunity and are much more prone to clinical infections on their return.[129] Another example of protective immunity comes from tick-borne relapsing fever, a disease caused by spirochaetes of the genus *Borrelia* that is transmitted by soft ticks in east Africa. Local people who have developed semi-immunity against relapsing fever are aware that scant contact with the soft-tick vector substantially increases their subsequent risk of contracting relapsing fever. Therefore, individuals leaving endemic areas traditionally carry soft ticks with them and let these ticks feed regularly to ensure their immune response to *Borrelia* spp is maintained (K Kurtenbach [deceased], personal communication). Both examples suggest that, contrary to common practice, non-treatment of asymptomatic multiple-strain infections might be better than treatment if parasite clearance reduces natural protection against more severe disease.

On the basis of the notion that different species cannot stably coexist if they occupy the same niche (the so-called competitive exclusion principle),[130] researchers proposed that superinfection with a competitively superior strain could be used to eliminate strains with antibiotic resistance.[131] However, as far as we are aware, competitive exclusion (ie, complete removal of one strain) has never been shown conclusively for pathogen strains.

Mutualistic pathogen–pathogen interactions

Many pathogens have immunosuppressive properties,[132] and by suppression of the immune system, one strain reduces the effect of immunity on others. The relative amount of suppression and density of the strains will determine how much different strains benefit.

Two further mechanisms involve the specific immune response. First, strain-specific pathogen epitopes can act as altered peptide ligands,[133,134] which inhibit the immune response to other epitopes of another strain. This process has been shown experimentally for two naturally occurring variants of a malaria cytotoxic T-cell epitope in *P falciparum*.[10] Each epitope substantially decreases the immune response against the other. As expected for a trait under strong natural selection, these two epitopes also arise together much more frequently than expected by chance.[135]

The second mechanism is known as original antigenic sin.[136] Here, epitopes of a strain erroneously trigger an immune response that was originally directed against another similar epitope and for which immune memory already exists. Production of antibodies against epitopes of the previously infecting strain blocks creation of antibodies specific to the new epitopes. The response is, therefore, less effective against the strain triggering it, indirectly benefiting that strain by comparison with the immune reaction that would have been stimulated in a naive host. This mechanism is usually invoked for sequential infections but it might also work in simultaneous multiple-strain infections, especially if one strain is initially much more abundant or grows much faster than the other. All mutualistic interactions between strains inhibit pathogen clearance and, therefore, are viewed as detrimental for the host.

Evolution of strain virulence and other pathogen traits

An understanding of evolutionary processes can greatly benefit clinical medicine.[137] Pathogen evolution is driven not only by fairly rare de-novo mutations but also by changes in allele frequencies in pathogen populations, and it can happen very quickly if selective pressures are strong. In pathogens, fast evolution is almost invariably the case because host immune responses and drugs impose strong selective pressure. Under such circumstances, large changes in the genetic structure of populations can arise within fewer than ten pathogen generations.[138,139] The spread of antibiotic resistance and its close correlation to the amount of antibiotics prescribed (selective pressure) is a prime example and of great concern to clinical medicine and public health.[11]

Theory predicts that competition between strains will cause individual pathogen strains to evolve towards higher virulence (defined here as the level of harm caused by a pathogen to its host).[140] This process takes place because most pathogens face a trade-off between their own replication in the host and host survival, which is vital for their transmission. If a host has one infection, selection will favour an optimum intermediate virulence that balances host exploitation with the chances of transmission. If more than one strain competes in the same host individual, selective pressures shift. Pathogen strains will be selected for higher resource use or faster growth, which increase their competitive ability and chance of survival and transmission relative to those of other strains (the so-called tragedy of the commons).[141] As a by-product,

virulence of pathogen strains will increase. This rationale is attractive and seems to be accepted widely, but no clear empirical evidence exists to causally link multiple-strain infections to virulence of individual strains. So far, research done in rodent malaria has shown that more virulent strains are competitively superior[6] and suffer less from competition,[142] but no data indicate that virulence of individual strains evolves in response to multiple-strain infections. The theory has been difficult to test because efficient ways to physically separate strains to measure changes of individual strains in response to co-infection are only available in a few systems.[109] Natural selection will favour adaptations that evade the host's immune response and convey competitive advantages over another strain, both of which will generally lead to negative effects on the host.

Pathogens can cooperate with each other to produce common goods necessary for population growth that are shared (eg, siderophores in bacteria).[4,143] Multiple-strain infections might select for so-called cheater genotypes, which exploit these common products without producing them themselves, thus lowering the total population growth rate and virulence.[144] Experimental evidence for this mechanism comes from work done in a phage[4] and the bacterium *Pseudomonas aeruginosa*.[143] Further experimental and observational data would be welcome. However, in these cases, virulence is defined as a feature of the pathogen population as a whole, not the individual strain, and the hypothesis does not predict higher virulence of individual strains.

For pathogen evolution, the proportion of host individuals infected by multiple strains (figure) could be less important than the proportion of strains that are exposed to others, which might have quite a different distribution, depending on the amount of aggregation of multiple infections. These data are hard to obtain, since they require very detailed analyses[16] of many host individuals.

Genetic exchange between pathogen strains

Because multiple-strain infections are a prerequisite for recombination among strains, they facilitate rapid generation of new variants that can evade drugs, vaccines, or the immune response. Since recombination also disrupts newly formed genotypes again, pathogens that recombine occasionally, but that mainly reproduce clonally, have the highest potential to evolve escape variants rapidly. The best known example is influenza A, in which reassortment among strains circulating in human and avian hosts is believed to cause antigenic shifts that lead to sporadic pandemics.[145] In *Vibrio cholerae*, recombination is thought to have a major role in creation of virulent strains from non-virulent environmental strains.[146] Sleeping sickness is another example of the importance of recombination. East African sleeping sickness is caused by *Trypanosoma brucei rhodesiense*, which is identical to the non-human infective *Trypanosoma brucei brucei* except for the presence of a serum resistance associated gene, *SRA*, which prevents lysis by human serum and confers human infectivity.[147] The two forms readily exchange genetic material in the laboratory.[148] Multiple infections enable introduction of *SRA* into *T b brucei*, transforming *T b brucei* into *T b rhodesiense*[149] and giving rise to new and potentially virulent *T b rhodesiense* strains. Multiple infections are therefore a key factor for the spread of east African sleeping sickness and creation of new pathogen strains.

For pathogens encountering treatment with several drugs, recombination facilitates emergence of strains resistant to several drugs by joining genes or alleles that confer resistance to one drug. The large genetic variation generated by recombination also enables evolution of more virulent variants. Horizontal gene transfer is very common in bacteria and has the same effects. For medicine, horizontal gene transfer is especially relevant because it enables transfer of resistance genes among both related and unrelated bacteria.[150]

Conclusions

Our Review provides strong evidence that multiple-strain infections are the norm, not the exception. Growing empirical evidence from patients and animal models shows that multiple-strain infections can change pathogen dynamics, disease course, and transmission.

For many human pathogens, we do not yet understand how their specific dynamics and effects on the host are altered by multiple-strain infections. Hypotheses have been proposed but, so far, have not been substantiated by empirical work, which would be of utmost basic and applied relevance. Multiple-strain infections are abundant and, for most pathogens, necessary methods are available to study them. Such research will help us to better understand pathogen and disease dynamics, to take optimum and effective measures against multiple-strain infections and, possibly, to tailor treatment strategies, and to predict future events that are linked to the interaction between strains, such as emergence of new recombinant strains and outbreaks of new variants.

Search strategy and selection criteria

To ascertain prevalence of multiple-strain infections, we searched PubMed (1950–2010) and ISI Web of Science (Science Citation Index Expanded, 1900–2010; Social Sciences Citation Index, 1956–2010; Arts and Humanities Citation Index, 1975–2010) with the keywords: "(multi[ple] OR dual OR double OR concurrent OR mixed) AND (strain OR genotype OR serotype OR serodeme OR zymodeme) AND infection(s)", "co-infection(s)", "superinfection(s)", and "polyclonal infection(s)". We also extracted relevant articles from reference lists of primary published work and from references received from colleagues. We only included publications that showed directly the occurrence of multiple strains of the same pathogen species in one host individual at the same time using reproducible direct genotyping methods (ie, not solely based on random amplification of polymorphic DNA, methods that are notorious for spurious results).

Contributors
OB had the idea for the Review and did the literature search. OB and MT discussed the key findings and wrote the Review.

Conflicts of interest
We declare that we have no conflicts of interest.

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