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The mathematics of random mutation and natural selection for multiple simultaneous selection pressures and the evolution of antimicrobial drug resistance

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The random mutation and natural selection phenomenon act in a mathematically predictable behavior, which when understood leads to approaches to reduce and prevent the failure of the use of these selection pressures when treating infections and cancers. The underlying principle to impair the random mutation and natural selection phenomenon is to use combination therapy, which forces the population to evolve to multiple selection pressures simultaneously that invoke the multiplication rule of probabilities simultaneously as well. Recently, it has been seen that combination therapy for the treatment of malaria has failed to prevent the emergence of drug-resistant variants. Using this empirical example and the principles of probability theory, the derivation of the equations describing this treatment failure is carried out. These equations give guidance as to how to use combination therapy for the treatment of cancers and infectious diseases and prevent the emergence of drug resistance. Copyright © 2016 John Wiley & Sons, Ltd.

Keywords: random mutation; natural selection; probability theory; combination therapy; drug resistance

1. The mathematics of drug treatment failure

In reference [1], 'The Basic Science and Mathematics of Random Mutation and Natural Selection', the governing equation describing the evolution of drug resistance to a single targeted selection pressure is derived. This equation shows that in order for a lineage in a population to evolve resistance to that targeted selection pressure, it must do so by a cycle of beneficial mutation followed by amplification of that mutation by repeated replications over generations in order for the probability to improve that another beneficial mutation will occur on some member of that lineage to improve fitness. This model of evolution differs from other models from evolutionary biology. This cyclical process of beneficial mutation and then amplification of beneficial mutation in order to improve the probability of the next beneficial mutation occurring on one of the variants is different than the evolutionary process discussed by Haldane in his classic paper *The Cost of Natural Selection* [2]. In this paper, Haldane proposes 'The principle unit process in evolution is the substitution of one gene for another at the same locus'. Kimura in his paper *On The Probability Of Fixation Of Mutant Genes In A Population* [3] uses a different mathematical approach but is conceptually using the same principle, which Haldane uses. Kimura models the fixation of a gene using a diffusion equation. This diffusion equation is very familiar to any student of conduction and convection heat transfer. Like Haldane's model, Kimura's model starts with a relative frequency for the gene to be fixed as 0, and that gene replaces other variants in the population until the relative frequency of that gene becomes 1.

There are two fundamental differences in Haldane's and Kimura's models used to model the mathematics of the evolutionary process when compared with reference [1]. The first difference is a physical difference. Natural selection is being modeled by Haldane and Kimura using a conservation

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principle. The increase of one variant in a population comes at the expense of the decrease of another variant in the population. Flake [4] showed in his analysis of Haldane's cost-of-selection model wrote the following.

> A quantity that is only dependent on the initial and final state of a system and is invariant to variations in the detailed motion between states is often associated in the physical sciences with a potential energy function.

Kimura introduces the same concept when he models fixation of a gene by selection using a diffusion rate equation. Natural selection does not operate as a conservation phenomenon. The increase in variants (amplification) in a given lineage does not have to be accompanied by a decrease in the other variants in a population. However, as Kimura does by dropping the transient term in his diffusion equation, he then can approximate the steady-state fixation of a gene in a population as a conservation phenomenon.

Weinreich in his publication *Darwinian Evolution Can Follow Only Very Few Mutational Paths to Fitter Proteins* [5] demonstrates empirically that natural selection does not operate as a conservation phenomenon when his lab subjected *Escherichia coli* to antibiotic selection pressure, which gave rise to multiple different lineages each following their own evolutionary trajectories in order to adapt to the antibiotic selection pressure. It is not substitution or fixation that determines the probability of the next beneficial mutation occurring on a lineage. Natural selection works by increasing the number of members in a lineage to improve the probability of the next beneficial mutation occurring on one of its members, but it does not have to occur at the expense of other variants disappearing.

A simple analogy can be used to compare Haldane's and Kimura's approach with the model presented in reference [1]. The analogy for Haldane's and Kimura's model consists of taking a deck of cards. Shuffle the deck and then randomly take 26 cards out of the deck. Those 26 cards are now out of the population. In the remaining 26 cards, double each of them and return the copies back to the deck. So if the Ace of Hearts was still in the deck, you now have two Aces of Hearts. If the Ace of Spades was selected out, you have no Ace of Spades in the population. Shuffle the deck and then again randomly remove 26 cards. Then duplicate the remaining 26 cards in the deck and return the doubled cards to the deck. If no Aces of Hearts were selected out, you would now have four Aces of Hearts in the deck. If there were two Kings of Clubs but one was selected out, you would still have two Kings of Clubs in the deck and so on. Repeating this process over and over of shuffling the deck and then removing half the deck would leave you with 52 copies of just a single card repeated. The process started with the relative frequency of each card was 1/52, and at the end of the process, the relative frequency of the remaining card value is $52/52 = 1$ (that is, the remaining card value has been 'fixed' or 'substituted' in the deck). This approach to the mathematics does yield a conservation model where the conserved value is the total population size; however, to correctly describe the random mutation and natural selection phenomenon requires a non-conservative model.

The non-conservative analogous card deck model consists of a standard 52-card deck. Selection is applied, and any of the variants killed or impaired from reproducing are removed from the deck. The remaining cards in the deck need to double over and over for many generations in order for there to be sufficient numbers and for there to be a reasonable probability that the next beneficial mutation in an evolutionary process occurs on one of its members. Substitution or fixation is not required for the evolutionary process to proceed. The only requirement is that a particular variant replicates sufficiently for the probabilities of another beneficial mutation occurring on one of its members.

The other difference in evolutionary biological mathematical models like Haldane's cost-of-selection and Kimura's fixation of gene models, when compared with non-conservation models, is that they do not take into account the multiplication rule of probabilities. Random mutation and natural selection is a stochastic process where replication is the random experiment and there are two possible outcomes. One possible outcome is that a mutation occurs at a particular site and the other possible outcome is the mutation that does not occur at the particular site. As long as mutations are random independent events, the joint probability of two or more beneficial mutations occurring in a lineage will be governed by the multiplication rule of probabilities. Neither Haldane's model nor Kimura's model takes this important mathematical fact into account in their models. It is the multiplication rule of probabilities that is the main governing mathematical principle, which determines whether an evolutionary process has a reasonable probability of occurring.

It is not very newsworthy these days when someone wins a lottery. Occasionally, though, one will see a news report about someone winning two lotteries. The fact that sometimes someone wins two lotteries

attracts attention. When treating infections and cancers, the selection pressures; that is, antibiotics, chemotherapy used to treat these diseases, often time fails because of the random mutation and natural selection phenomenon. Rare beneficial mutations give resistance to these selection pressures. In Ref. [1], the mathematics was derived showing how a lineage in a population can accumulate beneficial mutations to adapt to a single selection pressure targeting a single genetic locus. However, when the lineage is subjected to only a single targeted selection pressure at a time, these beneficial mutations do not have to occur simultaneously in a single replication in order to improve fitness. No member of the population must 'win' two lotteries simultaneously. These beneficial mutations accumulate in a cycle of beneficial mutation followed by amplification (increase in the number of members) of the beneficial mutation before the probabilities of the next beneficial mutation in the evolutionary sequence have a reasonable probability of occurring on one of those members. In other words, the multiplication rule of probabilities imposed by a sequence of binomial probability conditions is improved by natural selection by increasing the number of members who would benefit from a particular mutation in a cycle of beneficial mutation followed by amplification of that member who obtains the next beneficial mutation.

In the case where selection conditions target more than a single genetic locus simultaneously, this cycle of beneficial mutation followed by amplification of the beneficial mutation is disrupted. Even if some member of the population obtains a beneficial mutation for one drug, the other drug(s) impairs its ability to amplify and improve its probability to obtain the next beneficial mutation in an evolutionary sequence. However, under specific circumstances, populations can evolve to selection conditions targeting multiple genetic loci simultaneously. The mathematics, which governs these conditions, is derived here.

The derivation of the equations, which describe the evolution of drug resistance in the context of combination therapy, will be carried out using an empirical example. This example describes the emergence of drug-resistant malaria in the context of two-drug therapy. While this example is of particular importance to the use of selection pressures in the practice of treatment of malaria, the principle is more general and can be applied to the evolution of drug-resistant variants in the treatment of infectious diseases, herbicide resistant weeds, pesticide resistant insects, and failure of cancer treatments in the context of multiple simultaneous selection pressures.

The mathematical principles used to derive the equations of random mutation and natural selection are obtained from the text, *Advanced Engineering Mathematics* [6] by Erwin Kreyszig.

We start the derivation of the equations in the next section with the empirical example Ref. [7] of failure of the use of combination therapy to prevent the emergence of drug-resistant variants in the treatment of malaria.

1.1. An empirical example of drug treatment failure when using combination selection pressures

The empirical example that we will use to frame the derivation of the equations, which describe treatment failure when using combination therapy, was published in *Malaria Journal* and is titled *Failure of artesunate–mefloquine combination therapy for uncomplicated Plasmodium falciparum malaria in southern Cambodia* [7] and was written by William O Rogers, Rithy Sem, Thong Tero, Pheaktra Chim, Pharath Lim, Sinuon Muth, Duong Socheat, Frédéric Ariey, and Chansuda Wongsrichanalai. Included here is the abstract:

1.2. Abstract (background and conclusion) failure of artesunate–mefloquine combination therapy for uncomplicated Plasmodium falciparum malaria in southern Cambodia

1.2.1. Background. Resistance to anti-malarial drugs hampers control efforts and increases the risk of morbidity and mortality from malaria. The efficacy of standard therapies for uncomplicated *P. falciparum* and *Plasmodium vivax* malaria was assessed in Chumkiri, Kampot Province, Cambodia.

1.2.2. Conclusion. The results suggest that artesunate–mefloquine combination therapy is beginning to fail in southern Cambodia and that resistance is not confined to the provinces at the Thai-Cambodian border. It is unclear whether the treatment failures are due solely to mefloquine resistance or to artesunate resistance as well. The findings of delayed clearance times and elevated artesunate IC50 suggest that artesunate resistance may be emerging on a background of mefloquine resistance.

What this empirical example demonstrates is that despite the use of combination therapy, drug-resistant variants are emerging. The use of two drug simultaneous combination therapy is forcing the population of malaria parasites to take a more complex evolutionary trajectory than the simpler

evolutionary trajectories when the drugs are used sequentially. It is possible that variants already exist in some of these populations that are already resistant to one drug or another. After all, for example, mefloquine has been available since the 1970s, and resistance of malaria to this drug was already appearing in the 1980s [8] and not only has this drug been used for single drug therapy for the treatment of malaria but also has been used for many years as prophylaxis for malaria. This effectively would convert the combination therapy into sequential therapy where the remaining effective drug would only require the population to evolve to that single drug (a process describe in Ref. [1]). Then the process of beneficial mutation followed by amplification of the beneficial mutation cycle evolution of drug resistance could be followed by the population without the second drug disrupting the amplification process. For the sake of this discussion, we will assume that no drug-resistant variants exist in any of the subjects and explain mathematically how drug resistance can evolve de novo to two drugs simultaneously in a population.

Let us assume that drug resistance for the mefloquine requires mutations A1, B1, and C1 while drug resistance for the artesunate requires mutations A2, B2, and C2. The numerical modifiers indicate the particular drug, and the letters A, B, and C indicate the sequence in which the mutations must occur in order to have improved fitness for that particular drug. Then, any population of malaria, which has no resistance to either drug, must take a more complex evolutionary trajectory when the drugs are used simultaneously and then if the drugs were used singly and in sequence. The evolutionary trajectory for mefloquine would require a lineage to satisfy the joint probability condition $P(\text{A1})P(\text{B1})P(\text{C1})$ in order to adapt to this particular drug. The evolutionary trajectory for a lineage to adapt to the artesunate would require that lineage satisfies the probability condition $P(\text{A2})P(\text{B2})P(\text{C2})$ in order for that lineage to adapt to the other particular drug.

In order to evolve resistance to both drugs simultaneously, a lineage must satisfy the following probability condition, $P(\text{A1})P(\text{A2})P(\text{B1})P(\text{B2})P(\text{C1})P(\text{C2})$. Consider how the population may obtain a member with both the 'A1' mutation and 'A2' mutation simultaneously in order to produce a more fit member in the population. There are two random trials in this stochastic process as described in reference [1]. Those trials are the replication where a mutation occurs at a particular site or not, and the mutation itself is a random trial where of all the multiple possible mutations for this trial, only certain outcomes, are beneficial mutations. We describe the mathematics in the following section.

1.3. The mathematics of the empirical example of evolution of drug resistance with two targeted selection pressures

To obtain a better sense of the probability problem that we are addressing here, we can illustrate this with a Venn diagram (Figure 1). Subset A1 represents the members that obtained the A1 mutation in the single generation replication of the population. Subset A2 represents the members that obtained the A2 mutation in the same single generation of replication of the population. A1∩A2 is the set of members that obtained both the A1 and the A2 mutations in that generation.

To derive the mathematical behavior of the empirical example of the mutation and selection phenomenon, we first define some terms.

n – is the total population size

n_{A1} – is the sub-population size with mutation A1

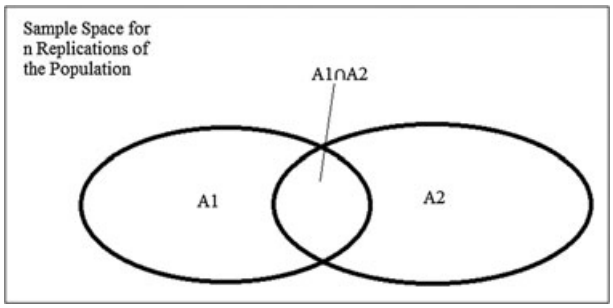

Figure 1. Sample space for the replication of the population size 'n' in a single generation with subset A1 (those members who obtain mutation A1), subset A2 (those members who obtain mutation A2), and the intersection of the two subsets A1∩A2 (those members who obtain both mutations A1 and A2).

n_{A2} – is the sub-population size with mutation A2

n_{A12} – is the sub-population size with both mutation A1 and A2

μ – the probability (frequency) that an error in replication will occur at a particular site in a single member in one replication

$P(\text{Beneficial}_{A1})$ – the probability that of all the possible mutations that can occur at the particular site that it will be the beneficial mutation A1

$P(\text{Beneficial}_{A2})$ – the probability that of all the possible mutations that can occur at the particular site that it will be the beneficial mutation A2

$P(\text{Beneficial}_{A3})$ – the probability that of all the possible mutations that can occur at the particular site that it will be the beneficial mutation A3

$P(A1)$ is the probability that beneficial mutation A1 will occur at a particular site, subscript 's' denotes in a single trial, subscript 'v' denotes a variable number of trials and subscript 'c' denotes the complement of $P(A1)$.

$P(A2)$ is the probability that beneficial mutation A2 will occur at a particular site, subscript 's' denotes in a single trial, subscript 'v' denotes a variable number of trials and subscript 'c' denotes the complement of $P(A2)$.

$P(A3)$ is the probability that beneficial mutation A3 will occur at a particular site, subscript 's' denotes in a single trial, subscript 'v' denotes a variable number of trials and subscript 'c' denotes the complement of $P(A3)$.

With these terms defined, we can determine the probability that mutation A2 will occur on some member of the population that already has mutation A1 in a single replication.

We start the computation with a population size 'n' of malaria parasites that replicates. Some of those members on replication will obtain mutation A1. Recognizing that the replication trial obeys the mathematics of the binomial probability distribution, using from reference [6], the definition of the mean for the binomial distribution is as follows:

$$n_{A1} = n^{*}P(\text{Beneficial}_{A1})\mu \tag{1}$$

where $P(\text{Beneficial}_{A1})$ has a value between 0 and 1 and represents of all the mutations that could occur at a particular site that it is the beneficial mutation. The value n_{A1} gives an estimate of the number of members in the population n will get mutation A1. We could easily compute the variance (and standard deviation) for this distribution and adjust the value of n_{A1} up or down to see how it affects the probabilities, but for this study, we will use the mean value. We know that from probability theory, the variance of a binomial distribution is as follows: $\sigma^2 = npq$ ref. [6], where n is the number of trials (replications for our case), $p = P(A1)_s$, $q = P(A1)_{c,s}$. Then the variance becomes $\sigma^2 = n * P(A1)_s * P(A1)_{c,s}$. But $P(A1)_{c,s}$ is very close to 1 for our example, and therefore, $\sigma^2 = n * P(A1)_s = n * P(\text{Beneficial}_{A1})\mu$, the mean value for our example. Even if we chose a population size that deviated from the mean value of the distribution by three standard deviations, our value for n_{A1} would only vary by $\pm 3(n * P(\text{Beneficial}_{A1})\mu)^{1/2}$. For a population size of $n = e12$ and $P(\text{Beneficial}_{A1})$ $\mu = e-6$, the standard deviation would be proportional to e3, less than a 1% error in our estimated sub-population size.

Then with this population n, the probability that at least one of those members who obtained mutation A1 is given in reference [1] Equation (14) and for this case appears as follows:

$$P(X) = \left(1-(1-P(\text{Beneficial})\mu)^{n^{*}n_G}\right) = P(A1)_v = (1-(1-P(\text{Beneficial}_{A1})\mu)^{n}), \text{ where} \tag{2}$$
$$X => A1,\ P(\text{Beneficial}) => P(\text{Beneficial}_{A1}),\ n = n,\ n_G => 1 \text{ generation}$$

Equation (2) is the probability that at least one member in the population obtained mutation A1 in the entire population in a single generation.

A plot of the values for Equation (2) that follows for various values of $P(\text{Beneficial}_{A1})\mu$ as a function of $P(A1)_v$ and n is shown in Figure 2.

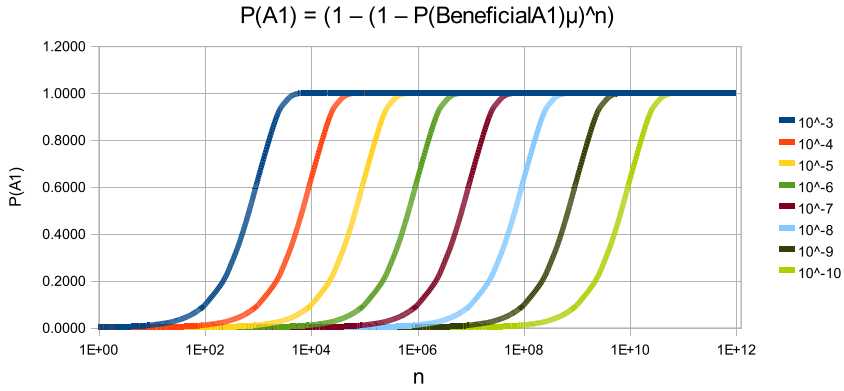

Figure 2. $P(\mathrm{A1})$ for various values of $P(\mathrm{BeneficialA1})\mu$ as a function of n. The color of the lines gives the value of $P(\mathrm{Beneficial_{A1}}) * \mu$.

Now, we need to compute the probability that at least a single member of that sub-population n_{A1} will also have mutation A2. We can again apply Equation (2) using Equation (1) to give us the size of the sub-population, which would benefit from mutation A2, and we obtain

$$P(\mathrm{X}) = \left(1-(1-P(\mathrm{Beneficial})\mu)^{n^{*}n_G}\right) = P(\mathrm{A2})_{\mathrm{v}} = (1-(1-P(\mathrm{Beneficial_{A2}})\mu)^{n_{\mathrm{A1}}}), \text{ where} \quad (3)$$
$$\mathrm{X} => \mathrm{A2},\ P(\mathrm{Beneficial}) => P(\mathrm{Beneficial_{A2}}),\ n => n_{\mathrm{A1}},\ n_{\mathrm{G}} => 1 \text{ generation}$$

Then the joint probability that an A2 mutation will also occur on some member that had an A1 mutation is given by the multiplication rule and yields:

$$P(\mathrm{A1})_{\mathrm{v}}P(\mathrm{A2})_{\mathrm{v}} = (1-(1-P(\mathrm{Beneficial_{A1}})\mu)^{n})(1-(1-P(\mathrm{Beneficial_{A2}})\mu)^{n_{\mathrm{A1}}}) \quad (4)$$

The value for n_{A1} is given by Equation (1). If we let $P(\mathrm{Beneficial_{A2}})\mu = P(\mathrm{Beneficial_{A1}})\mu = P(\mathrm{Beneficial})\mu$, Equation (4) becomes:

$$P(\mathrm{A1})_{\mathrm{v}}P(\mathrm{A2})_{\mathrm{v}} = (1-(1-P(\mathrm{Beneficial})\mu)^{n})(1-(1-P(\mathrm{Beneficial})\mu)^{n_{\mathrm{A1}}}) \quad (5)$$

A graph of Equation (5) for various values of $P(\mathrm{Beneficial})\mu$ is shown in Figure 3.

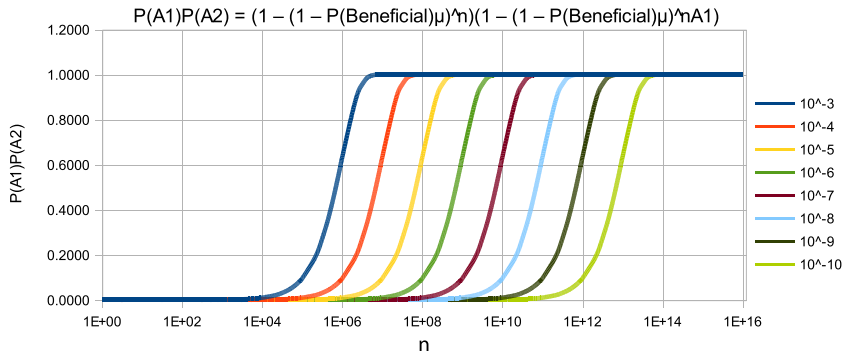

Figure 3. $P(\mathrm{A1})P(\mathrm{A2})$ for various values of $P(\mathrm{Beneficial})\mu$ as a function of n. The color of the lines gives the value of $P(\mathrm{Beneficial}) * \mu$.

1.4. The mathematics of the empirical example of evolution of drug resistance with three targeted selection pressures

Consider the condition where three targeted selection pressures are used where selection pressure '1' requires mutations A1, B1, and C1, selection pressure '2' requires mutations A2, B2, and C2, and selection pressure '3' requires mutations A3, B3, and C3 in order to adapt to these conditions and mutations A1, A2 and A3 must occur simultaneously in order to improve fitness to replicate.

The Venn diagram for this problem appears as shown in Figure 4:

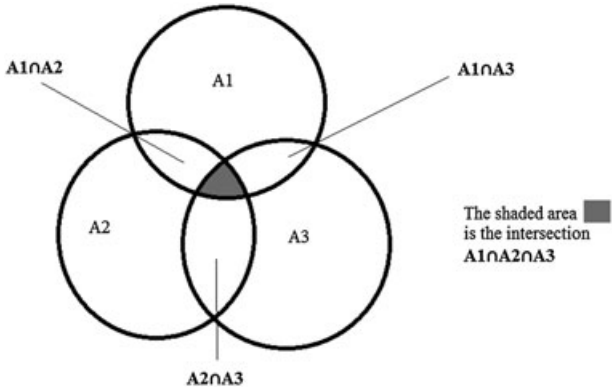

Figure 4. Sample space for the three selection pressures subset A1, subset A2, subset A3, and the intersection of the three subsets A1∩A2∩A3 shaded.

Assume that the first selection pressure requires mutations A1, B1, and C1, the second selection pressure requires mutations A2, B2, and C2, and the third selection pressure requires mutations A3, B3, and C3 in order for a member to adapt to these selection conditions, and it requires that mutations A1, A2, and A3 occur simultaneously in order to improve fitness to replicate. That condition corresponds to (A1∩A2)∩A3. The probability in the region given by (A1∩A2) has already been computed and given by Equation (5). In order to compute the joint probability (intersection) of the (A1∩A2) region, we need to know the number of trials (replications) occurring on members with both the A1 and A2 mutations.

In order to compute the probability that at least a single mutation A3 will occur on some member of the sub-population with both mutations A1 and A2, we need to estimate the size of that sub-population. We can again use Equation (1), the mean value of the binomial distribution to estimate the size of the sub-population with both mutations A1 and A2.

$$n_{\mathrm{A12}} = n^{*}P(\mathrm{beneficial}_{\mathrm{A1}})\mu^{*}P(\mathrm{beneficial}_{\mathrm{A2}})\mu = n_{\mathrm{A1}}{}^{*}P(\mathrm{beneficial}_{\mathrm{A2}})\mu \tag{6}$$

With the value of n_{A12}, we can compute the probability that at least a single A3 mutation will occur on a member of that sub-population, which has both mutations A1 and A2. Using Equation (3), we obtain

$$P(\mathrm{X}) = \left(1-(1-P(\mathrm{beneficial})\mu)^{n^{*}n_{G}}\right) = P(\mathrm{A3})_{\mathrm{v}} = (1-(1-P(\mathrm{Beneficial}_{\mathrm{A3}})\mu)^{n_{\mathrm{A12}}}),\ \text{where}$$
$$\mathrm{X} => \mathrm{A3},\ P(\mathrm{beneficial}) => P(\mathrm{Beneficial}_{\mathrm{A3}}),\ n => n_{\mathrm{A12}},\ n_{\mathrm{G}} => 1\ \text{generation} \tag{7}$$

Then the joint probability that an A3 mutation will also occur on some member that had an A1 and A2 mutations is given by the multiplication rule and yields

$$P(A1)_{v}P(A2)_{v}P(A3)_{v} = (1-(1-P(Beneficial_{A1})\mu)^{n})(1-(1-P(Beneficial_{A2})\mu)^{n_{\mathrm{A1}}})(1-(1-P(Beneficial_{A3})\mu)^{n_{\mathrm{A12}}}) \tag{8}$$

The value for n_{A12} is given by Equation (6).

If we let $P(\mathrm{Beneficial}_{\mathrm{A1}})\mu = P(\mathrm{Beneficial}_{\mathrm{A2}})\mu = P(\mathrm{Beneficial}_{\mathrm{A3}})\mu = P(\mathrm{Beneficial})\mu$, Equation (8) becomes

$$P(A1)_{v}P(A2)_{v}P(A3)_{v} = (1-(1-P(Beneficial)\mu)^{n})(1-(1-P(Beneficial)\mu)^{n_{\mathrm{A1}}})(1-(1-P(Beneficial)\mu)^{n_{\mathrm{A12}}}) \tag{9}$$

A graph of Equation (9) for various values of $P(\text{Beneficial})\mu$ is shown in Figure 5.

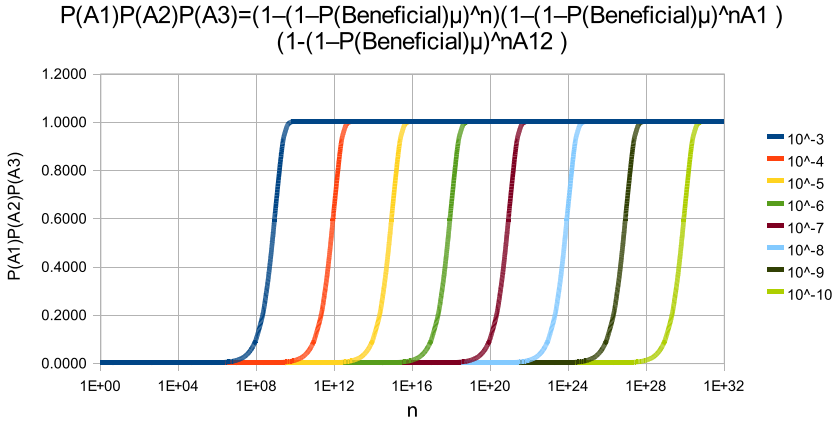

Figure 5. $P(\text{A1})P(\text{A2})P(\text{A3})$ for various values of $P(\text{Beneficial})\mu$ as a function of n. The color of the lines gives the value of $P(\text{Beneficial}) * \mu$.

1.5. Discussion of the mathematics of mutation and selection for multiple simultaneous selection pressures

A pattern emerges when doing the mathematics of random mutation and natural selection for multiple selection conditions. The first selection condition imposes a mean value for the number of beneficial mutations given by the mean value for a binomial distribution for the entire population. The second selection pressure, however, must have its beneficial mutation occur on a member with the first beneficial mutation. This subset of the population is much smaller than the entire population and is the mean value of the binomial distribution of that subset. When a third selection pressure is imposed simultaneously on the population, this beneficial mutation must occur on a sub-population, which already has mutations for the first two selection conditions, which is the mean value of the binomial distribution of that even smaller subset. The sub-population size for multiple beneficial mutations is the mean value of the binomial distribution for the subset of the population with the other beneficial mutations. This mathematical relationship is expressed as follows:

$$n_s = n_* \prod_{i-1}^{s} P(Beneficial_{Ai})\mu \tag{10}$$

Where n_s is the sub-population size for the sth selection pressure. The probabilities for these subsets of the population also show a pattern and are given by the following:

$$\prod_{i-1}^{s} P(A_i) = (1-(1-P(Beneficial_{Al})\mu)^n) \prod_{i-2}^{s} (1-(1-P(Beneficial_{Ai})\mu)^{n_{Ai}}) \tag{11}$$

Equations (10) and (11) give the sub-population sizes and probabilities for the first set of mutations (A_i) required to improve fitness for a set of simultaneous selection pressures. The equation for the second set of mutations (the B_i mutations) is analogous to Equations (10) and (11) except B_i would substitute for A_i. In addition, the initial population 'n' is determined by the amount the lineage with the A_i mutations can amplify. The progenitor for the population with the A_i mutations that would benefit from the B_i mutations must first amplify sufficiently so that there are a huge number of members that the nested binary probability process could occur again. A cycle of beneficial mutations followed by amplification of the beneficial mutations must occur by natural selection for there to be a reasonable probability that the next set of beneficial mutations occurs at the correct sites in the genome.

Equations (10) and (11) give rise to specific requirements for an evolutionary process by random mutation and natural selection where multiple simultaneous beneficial mutations are needed to improve fitness to have a reasonable probability of occurring. n_{A1} must be a very large number. This, in turn, requires that the population size n must be a huge number. Equation (1) gives that $n_{A1} = n^* P(\text{Beneficial}_{A1})\mu$ or $n = n_{A1}/P(\text{Beneficial}_{A1})\mu$. We can apply Equation (5) and the graph of Equation (5) in order to estimate the probability that a double beneficial mutation will occur in our empirical example, Ref. [7]. In Ref. [7], the authors studied subjects with parasitaemia between 1000 and

100,000 parasites/mm^3. In Ref. [8], we can obtain an estimate of the total parasite load based on the level of parasitaemia. In areas of endemicity, it is not uncommon for an infected person to be carrying more than 1×10^9 parasites (a child with an unremarkable parasite density of $1000\,\mu L^{-1}$ in the blood would have approximately this number), and such infections would be likely to contain at least one parasite with a point mutation at almost any nucleotide position (ignoring strongly deleterious mutations that might prevent development or replication). In Ref. [8], we also obtain a value for the mutation rate:

> Mutation in malaria parasites occurs at a fairly typical rate for a eukaryote (a point mutation rate of approximately 1×10^{-9} per nucleotide site per mitotic division), so new mutants are produced all the time in natural populations.

One study even provides evidence suggesting that some *P. falciparum* clones could have a higher mutation rate (potentially an adaptive 'mutator' phenotype). Thus, the total parasite load 'n' for the subjects from [7] could range between e10 to e12. Multiplying these values by e − 9, the mutation rate, we obtain a value for n_{A1} between e1 and e3. If we use these values to estimate the probability that a double beneficial mutation will occur on at least one member of the sub-population is clearly very low for the lower estimate but for the higher estimate, the probability is greater than 0.5. In addition, this shows that the probability of a double beneficial mutation occurring is very sensitive to the value of the mutation rate used. The mutation rate affects the sub-population size on which the double beneficial mutation would occur as well as the particular probability curve used to estimate the probability. References [9], [10], and [11] suggest that the mutation rates increase for the malaria parasite when under selection pressure. If the mutation rate of e − 8 is used instead of e − 9, the value for n_{A1} is then between e2 and e5. Using the lower estimate of the e − 8 curve, the probability of a double beneficial mutation occurring is about 0.1. Using the upper estimate for the sub-population size shows that the probability is essentially 1 that a double beneficial mutation will occur. And if you have millions of people suffering from malaria, even a low frequency (probability) that a double beneficial mutation occurring becomes a real possibility of occurring in one or more of the people suffering from malaria. Once that double beneficial mutation A1 and A2 occurs, if that variant can amplify, then the process can repeat itself for the B1 and B2 mutations, which when these mutations occur and can, in turn, amplify leading to the C1 and C2 mutations occurring. Again, we see a cycle of multiple beneficial mutations occurring simultaneously followed by an amplification phase in order to improve the probability of more multiple simultaneous beneficial mutations occurring. Another consideration is the total population size used in the calculation. From [7], we obtained a total parasite load, 'n', of e10 to e12. However, in [12], these researchers report parasitaemia at much higher rates. In this study, they consider cases of hyperparasitaemia between 4–75% of red blood cells infected. In a figure from this study, they show a single red blood cell from a blood smear with as many as 10 parasites in a single cell. In this case, the total population of parasites will be at least one to two orders of magnitude higher than those in [7]. Under these circumstances, it is very likely that double beneficial mutations will occur and if these variants with double beneficial mutations can amplify, then drug resistance to two drugs will occur by an accumulation of double beneficial mutations by a cycle of double beneficial mutations and amplification of the double beneficial mutations.

What the previous calculation shows is that durable treatment for malaria will most likely require three-drug therapy because of the huge populations of parasites and the large number of people subject to malaria infections. The same concept can be applied to the treatment of any replicator causing disease. The clinical physician can estimate the size of the population of replicators (bacteria, virus, parasite, and cancer cell). Then the clinician can examine Figures 2, 3, or 5 and determine the probability that resistant variants exist in the population for 1, 2, or 3 selection pressures.

As a specific example of how to use the previous calculation for the field of oncology, radiological studies can be carried out to estimate the size of a tumor. A pathologist can do histological studies of the tumor and determine the number of cancer cells per volume and from the total size of the tumor and the number of cancer cells per volume, the total number of cells can be computed. This total number of cells would give guidance in the number of targeted selection pressures necessary in order to have a reasonable probability of driving the cancer to extinction.

2. Conclusions

In Ref. [1], it was shown that the random mutation and natural selection phenomenon when acting with a single selection pressure operating on a single genetic locus work by a sequential cycle of beneficial

mutation and amplification of that beneficial mutation in order to overcome the multiplication rule of probabilities for joint beneficial mutations to occur on a lineage. The evolutionary trajectory for this case consists of a sequence of single binomial probability problems. When multiple selection pressures are applied to a population simultaneously, the beneficial mutation and amplification of that beneficial mutation cycle are disrupted. However, under specific mathematical conditions, very large populations can still evolve to multiple selection conditions simultaneously. These populations evolve to these selection pressures by getting multiple beneficial mutations simultaneously. In the case of multiple simultaneous selection pressures, the mathematics consists of nested binomial probability problems where each set of nested binomial problems is separated by an amplification step.

The emergence of drug-resistant malaria has many potential causative factors. Some of these factors include patient compliance, patient immune status, and a variety of other factors. But if we ignore these factors and consider how large of an infecting population is required to give a reasonable probability of a double beneficial mutation occurring on a single replication, existing data already reveal that the probability of a double beneficial mutation to two simultaneous selection pressures is in the realistic range. For long-term suppression of emergence of drug-resistant variants in the treatment of malaria, it is most likely going to require three-drug therapy. The probability calculation for the three-drug 'cocktail' is carried out in the same manner as the two drug therapy. One would have three selection conditions imposing a much more complex evolutionary pathway when used simultaneously rather than three simpler evolutionary pathways if the drugs were used sequentially. The multiplication rule of probabilities would require the satisfaction of the probability $P(\mathrm{A1})P(\mathrm{A2})P(\mathrm{A3})$ in order to improve fitness for that variant. This also is the mathematics that governs the success of HIV treatment.

It is quite likely that any epidemic caused by replicators that can achieve huge populations in every infected individual will likely require three-drug combination therapy to control the epidemic and impair emergence of resistance. Resistance is already appearing to anti-influenza medications [13], and if a worldwide epidemic of influenza occurs similar to the episode of the early 1920s, which is resistant to existing drugs and effective immunization is not available, the medical system will not be prepared to deal with such an occurrence.

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