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"The Wild, the Watery, and the Unshored" — Antimicrobial Resistance Goes To Sea

Basil P. Tangredi, DVM
Richard H. Evans, DVM, MS, FNAP
Dennis F. Lawler, DVM, FNAP
Pacific Marine Mammal Center, 20612 Laguna Canyon Road, Laguna Beach CA 92651

Bacterial resistance to antibiotics (AR) is a global crisis that is placing nearly every ecological niche at risk for becoming an AR reservoir (Alves 2014). Biomedical literature is replete with studies of pharmaceutical effects on microbial populations, and researchers are beginning to take a broader view of the concerns with finding AR in many bacterial species in diverse ecologies.

Increasingly, AR in a given isolate involves multiple antibiotics or classes of antibiotics. Medical practices and other human activities clearly play prominent roles in the surprising, and even shocking, extent of AR dissemination (Hawkey and Jones 2009). Woolhouse and Ward (2013), looking at the source scope of environmental activities that can lead to accumulated AR genes, point to livestock-derived foods; animal and human wastes; animal wastes used as fertilizers that in turn contaminate food and water supplies; direct contact among humans and animals; lack of sanitation in local-to-regional human habitats, and 'imported' foods. The marine environment can be added as yet another victim (and source) of AR contamination.

Assessing AR patterns of marine infectious organisms is one means of monitoring effects of changing ocean biology on animal and human health. Our study was conducted at Pacific Marine Mammal nter (PMMC), which for 30 years has served the ocean off Laguna Beach CA USA under a permit from NOAA. This aquatic region features typical cold southern CA waters, and currents teeming with plankton that feed large numbers of the small fish that in turn sustain moderate numbers of seals on and around a group of small coastal islands (Channel Islands).

We report and discuss results of a 10-year survey of bacterial species and AR from seals that presented to PMMC Laguna Beach.

How was the study conducted?

At PMMC, each presenting injured or stranded pinniped or cetacean is given a physical examination, and appropriate additional diagnostic procedures are conducted. The determinant for including bacterial culture and sensitivity in the diagnostic process usually involves cutaneous–subcutaneous abscesses, thoracic or abdominal effusion, or unusual pathologies such as ocular or nasal discharge and recognized organopathy. Culture and evaluation of bacterial isolates for resistance patterns are conducted by IDEXX Laboratories, Los Angeles, CA, USA.

The data supporting the analyses and interpretations of bacterial culture and AR were collected between 2004 and 2013. The seal species included in study are the California sea lion (*Zalophus californianus)*, harbor seal (*Phoca vitulina*), and northern elephant seal (*Mirounga angustirostris*).

We first tabulated bacterial isolates in our database by source-within-individual. Abdominal source isolates were organ, urine, or effusion-derived; thoracic isolates were tissue (lung, heart blood, trachea)- or effusion-derived; surface isolates were forequarter-, hindquarter-, or flipper-derived; head (includes ocular-nasal discharges, oral lesions, submandibular lymphadenopathy); central nervous system (brain and spinal cord). Isolate frequencies were evaluated across sub-categories. When Chi-square analyses revealed no statistically significant differences ($P>0.05$) among sub-categories, the data were grouped into primary categories of abdominal, thoracic, or body surface sites. This combined database was evaluated based on therapeutic relevance that attributes antibiotic resistance to a pharmacological class if at least one antibiotic in that group is resisted. We used the multidrug resistance (MDR) and extensive drug resistance (XDR) format proposed by Magiorakos and colleagues (2012) to evaluate isolate AR using seven antibiotic categories and sentinel antibiotics.

For the analyses, we included "intermediate resistance" isolates to a given antibiotic with "resistant" isolates, and described this group as "non-susceptible". Isolates in this group were non-susceptible to at least one antibiotic in a given class (Table 1). Multi-drug resistance (MDR) is non-susceptibility to 3 - 4 antibiotic classes. Extensive drug resistance (XDR) is non-susceptibility to 5 - 6 antibiotic classes. Pan-drug resistance (PDR) is non-susceptibility to all antibiotic classes (Magiorakos et al 2012). One caveat in this scheme is that every known antibiotic was not tested. Thus, it is appropriate to use the qualifiers suspected-MDR, suspected-XDR, and suspected-PDR, although we use the abbreviated forms in the text here, for simplicity.

We defined "environmental" bacteria (E) as species likely to be acquired from the human-dominated microbiological environment. We defined "marine" bacteria (M) as species that are endogenous to marine mammals and their natural food chain. Isolates that were identifiable only to genus or as "gram-negative rod" (example) were included with the appropriate associated environmental gram-stain group: that is, "by the company that they keep."

We performed two tests on data that were organized by year of collection (2004 – 2013). As a test for independence, a chi-square metric measured how far observed isolate counts departed from expected counts under the null hypothesis; the *p*-value answers the question of what chance existed that random sampling would yield an equal or stronger association among results. The categorical variables (host species, anatomical location, and E vs M) were analyzed in this fashion.

Trend analyses were necessary because of yearly variation in isolate numbers. A linear trend analysis fits a straight line to the yearly data and the fraction of non-susceptible isolates, wherein a slope that differs significantly from zero defines time-associated relationships in the data set. The test for trend also yields a χ^2 statistic and a *p*-value. If a large proportion of the variation across the years is due to a trend, then the *p*-value will be small.

What were the analytical outcomes?

Among total isolates from the three host seal species, 45% of California sea lion isolates, 45% of elephant seal isolates, and 48% of harbor seal isolates, were non-susceptible ($\chi^2 = 0.09$, p=0.96). Non-susceptible bacterial isolates did not segregate by host species.

Percentages of isolates of non-susceptible bacteria from anatomical locations were: Abdomen 48%; thorax 43%; head 45%; skin 45%; central nervous system 46%. There was no difference in non-susceptibility among isolates, based on anatomical origin ($\chi^2 = 1.1$, p=0.89). Numbers of isolated bacterial species (E or M) differed strongly ($\chi^2 = 68$, p<0.0001), with 50% of E isolates and 4% of M isolates being non-susceptible.

Among all 925 bacterial isolates, 74% were E-Gram-Negative; 16% were E-Gram-Positive; 4% were M-Gram-Negative; 6% were M-Gram-Positive. Non-susceptibility occurred in 419 (45%) of isolates: 60% of non-susceptible isolates were MDR [n=257; 27% of *all* isolates]; 40% of non-susceptible isolates were XDR [n=167; 18% of *all* isolates]. For the predominating E-Gram-Negative group, independence testing yielded $\chi^2 = 53.2$; $p = <0.0001$, and trend analysis yielded $\chi^2 = 31$; $p = <0.0001$. For yearly data of *all* non-susceptible isolates, $\chi^2 = 18.2$; $p = 0.03$, and trend analysis yielded $\chi^2 = 5.8$; $p = 0.016$.

Escherichia coli accounted for 33.0% of E-Gram-Negative isolates; 53.0 % were MDR/XDR. Non-susceptible *E. coli* accounted for 31.0% of all E-Gram-Negative non-susceptible isolates. Independence testing yielded $\chi^2 = 28$; $p = 0.001$; trend analysis yielded $\chi^2 = 19.5$; $p<0.0001$ (years 2008-2013 because of low sample sizes in other years). The proportion of non-susceptible isolates, and the yearly trend toward an increasing proportion of non-susceptible isolates, predominated in the data set (Figure 3).

Klebsiellae accounted for 11.0 % of E-Gram-Negative isolates, with 60.0% being MDR/XDR. Non-susceptible Klebsiellae accounted for 12.5% of all E-Gram-Negative non-susceptible isolates. Over the years 2008-2013, an alarmingly high proportion of *Klebsiella* isolates were non-susceptible, with no yearly trend.

Enterococci accounted for 50.0% of all E-Gram-Positive isolates, with 25.0% being MDR/XDR. Non-susceptible Enterococci accounted for 61.0% of all E-Gram-Positive non-susceptible isolates. Chi-square analysis was not valid because of low numbers in some categories, but non-susceptible Enterococci numerically dominated the field of non-susceptible E-Gram-Positive isolates.

Do other studies of AR dissemination help characterize the present magnitude of the problem?

A 1974 study of bacterial isolates from free-ranging California sea lions revealed generally high bacterial susceptibility to tetracycline, nitrofurantoin, cephalothin, chloramphenicol, ampicillin, and gentamycin (Sweeney et al 1974). During 1994-1995, investigators evaluated bacterial culture and antibiotic sensitivity of abscesses in California sea lions, harbor seals, and northern elephant seals (Johnson et al 1998). A majority of isolates (72%) were Gram-negative, with *E. coli* being the most frequent isolate; most isolates possessed multiple AR. The data thus are similar to our observations. The generally increasing AR between 1974 and 1995 is noteworthy, even though study structures were not identical.

A recent report looked at 46,921 Gram-negative and 19,174 Gram-positive isolates that had been collected in 26 European countries between 2004– 2010 (Denis et al 2014). The time period closely matched the time period of our study. In that study, extended spectrum β-lactamases (ESBL) were found in *Klebsiella pneumoniae* and *E. coli*, demonstrating that the AR phenomenon now is widespread in Europe, across geography and cultures.

A study in the Netherlands, a country that limits antibiotic usage and displays lower rates of AR generally, suggested that the high use of antibiotics in poultry-rearing in that country has facilitated movement of ESBL genes and plasmids, and *E.coli,* though the food chain from the poultry farm to humans (Leverstein-van Hall et al, 2011). Thus, even focused use of antibiotics outside the therapeutic arena appears to carry significant risk of spreading AR.

In fact, the alarming world-wide spread of AR has been under scientific scrutiny for some time, but precious little action has been taken. *Can* corrective action be taken? Kennedy (2013) indicated that 73% of antibiotic sales in the USA are related to livestock-rearing practices, supporting that farming practices are at least partly responsible for the rise of AR (Kennedy 2013). And, it is fair to suggest that climate change likely will increase production animal crowding, thus increasing environmentally-mediated stress and promoting transmission of infectious diseases. Thus, the opposing forces of food production and a looming AR crisis are evident and are causes for conflict, at least in the USA.

Heavy metals–antibiotics synergism is another agriculture consequence that impacts AR. In a recent report, interactions among a sub-therapeutic antibiotic presence and environmental metals such as silver, copper, and arsenic, appears facilitate a selective advantage for bacteria carrying multi-resistance plasmids, adding further complexity to the biology of AR dissemination (Gullberg et al, 2014) .

A further agricultural complication is that the monetary price for sweeping changes will be high, even though sweeping changes appear to be biologically necessary. Laxminarayan (2014) pointed out the options to use vaccination more effectively; increase efforts at disease control; employ more properly-timed use of disease diagnostics, focus public education, and perhaps incentivize these alternatives (Laxminarayan, 2014). In our view, this perspective is correct, but it is clear that agricultural re-planning at all levels will require allowance for the high costs associated with accomplishing the objectives.

On the other hand, the ultimate monetary costs of not re-thinking food production may be much greater. Pappas (2011) discussed the genetic context of selection pressures that result from sub-therapeutic antibiotic use in agriculture, citing resultant AR genes that are shed with bacteria in animal wastes, contaminating the environment and the food chain, and contributing to spread of zoonoses.

Venturini and colleagues [Venturini et al 2010] reported an enteropathogenic *E. coli* strain plasmid that carried genes for both antibiotic resistance and virulence, suggesting that co-selection for these two traits is occurring and increases risks for development of infectious organisms that are both highly virulent and drug-resistant.

Even this brief review reveals that warnings about AR dissemination and its consequences have been visible and audible for an extended period of years.

How did AR reach a near-crisis state?

Are agricultural conflicts the primary driver of AR dissemination, or are they parts of a larger environmental mess? Available information indicates that developing of AR is not a recent phenomenon (Tadessee et al 2012). Degrees of AR probably occur naturally, as normal biological sequelae to antibiotic development and usage paradigms, likely facilitated by human errors (Tadessee et al 2012). Is the genesis of the crisis closer to home? Bacteria in the intestinal tracts of humans and animals constantly exchange AR genes, not only among themselves as normal gut inhabitants, but also with bacteria that are "just passing through" (Salyers et al 2004). The latter may include environmental or oropharyngeal bacteria that are swallowed, and bacteria in water supplies or in various foods (Salyers et al 2004). A potential complication of this bacterial admixture process is that intestinal bacterial send molecular signals into the body, evidently as physiological health modulators (Wang et al; 2007; Richards et al 2008). A related question that needs to be explored further is whether bacterial exchanges of AR genes alter those signals in ways that influence organism health.

Forsberg and colleagues (2012) observed that extensive interspecific exchange of AR genes affects even soil microbes, indicating that the genomes of soil saprophytes and pathogenic bacteria are not truly distinct and emphasizing the importance of environmentally-maintained AR.

An interesting biological phenomenon is illustrated by the many species of *Shewanella* that are biodegrading organisms. Shewenellae are frequently isolated from marine invertebrates (Ivnova et al 2003). Perhaps as a parallel observation, five of seven *Shewanella* isolates in our database were resistant to cephalexin but otherwise displayed broad antibiotic susceptibility. Martinez (2008) suggested that bacterial genomic material may have evolved to protect against nonspecific toxic effects of ions and molecules in the natural environment, and that these genes were able to be "recruited" to act against antibiotics for this reason. Thus, the sometime-heard assertion that AR DNA originated "spontaneously" in soil may be at least partially misleading.

What pressures influence extensive AR gene dissemination into aquatic environments?

Baquero and colleagues (2008) described genetic reactors in AR dissemination: Human & animal microbiota; crowding that increases genetic exchange among bacteria; waste water and biological residue; soil surface and groundwater. Several specific examples include industrial antibiotics in water-sludge and soil-water; water disinfection and cycling, wastewater, and fertilizers (Baquero et al 2008).

Despite all of this activity, it is interesting that (long term) horizontal transfer of AR genes has not reduced bacterial diversity (Salyers et al 2004). Allen and colleagues (2010) review microorganism journeys that include physical forces (wind, watershed, blown dust, regional-to-intercontinental movement) creating many-sourced bacterial populations on land, in fresh water, and in marine environments. Animal transport of bacteria, biologically or mechanically, involves small rodents to large wild ruminants, primates, and especially bird species that migrate very long distances. Thus, even geographically isolated human and animal bacterial populations can acquire AR genes by contacts following long-distance transport events (Allen et al 2010).

How does human waste water contribute to disseminating AR bacterial genes in fresh water and marine environments?

In an early study, Cooke (1976) evaluated a New Zealand waste water treatment site, finding few fecal coliforms at a projected effluent water outlet, prior to opening the plant. Two years later, 72.8% of fecal coliforms in effluent displayed AR, often multiple. Even more disconcerting was that sediment filter-feeding shellfish concentrated fecal coliforms, showing 88.7% AR. The author suggested that, even at that early time, resistant human fecal coliforms may have a selective advantage in the water environment (Cooke 1976).

Australian investigators evaluated waste water AR *E. coli* from treatment effluent along the East-Central Australian coast, based on concern that proximate surface waters and oysters could be affected by waste processing (Watkinson et al 2007). AR *E. coli* were present, but were fewer (59% of isolates) than in some other studies, reflecting different regional medical and agricultural practices and amount of fecal pollution (waste treatment efficiency). Oyster contamination was observed, but also was lower than expected. The observations suggest that local practices in human cultures can affect AR in waste effluent, and secondarily in the environment (Watkinson et al 2007). Clearly, human motivation is very important in local management of bacterial environmental contamination problems.

Amos and colleagues (2014), evaluating waste effluent into UK rivers, report finding the AR resistance gene $bla_{\text{CTX-M-15}}$ in *E. coli* ST131. The investigators indicate that this gene codes for resistance to 3rd generation cephalosporin antibiotics, and is common in resistant *E. coli* and Klebsiellae. Thus, bacteria carrying this important gene are not being neutralized by rigorous waste treatment (Amos et al 2014), and humans should be aware of potential municipal sanitation failures.

Brechet and colleagues (2014), in a French urban setting, used a multifocal sampling protocol to evaluate waste effluent for total and ESBL *E. coli*. Alarmingly, the investigators found that the treatment process, by eliminating 98% of all *E. coli* and 94% of ESBL *E. coli*, actually increased the relative yield of ESBL-carrying organisms. Sadly, the resulting sludge is used as fertilizer, thus facilitating contamination of agricultural environments and the human food chain (Brechet et al 2014).

Studies conducted in different parts of the world and examined as a collection show that humans can influence dissemination of bacterial AR genes either positively or negatively. The weight of that influence appears to be negative, indicating that corrective actions of various types are needed urgently.

What is the role of birds in contaminating the marine environment?

A number of studies suggest that birds, especially migratory birds, may play a far more important world-wide role in dissemination of AR than may have been supposed. In fact, their role may be a primary driving force.

An AR study conducted along coastal North America from Kent Island Canada to Virginia USA (2005-2007) involved sampling 165 animals of 15 marine species, 192 animals of 15 bird species, and 13 animals of 3 shark species (Bogomolni et al 2008). Among 797 bacterial isolates, 73% displayed AR to 1-4 drugs and 27% displayed AR to 5-13 drugs. Gram-negative isolates predominated at 76% and *E. coli* strains were the most frequent isolate. The same group later reported on 472 bacterial isolates from 149 animals off the northeastern coast of the USA, demonstrating 58% AR overall, with 43% multiple AR. For the latter study, bacterial culture specimens were acquired from 79 sea birds (n=280); 64 marine mammals (n=174); and 6 sharks (n=11). Overall, cultures from sea birds demonstrated greater bacterial AR, and the authors indicated that sea birds should be further investigated as possible reservoirs for AR bacteria (Rose et al 2009).

Herring gulls (*Larus argentatus*) were evaluated for AR in *E. coli* from fecal cultures (Smith et al 2014). These gulls migrate long distances across Canada, the USA, and the Caribbean, and could contaminate environments along the way. In this study, 87% of gull samples harbored ESBL *E. coli*. The authors point out that, while not all of these isolates may be pathogenic for all species, birds clearly are extensively involved in AR dissemination.

Alves and colleagues examined AR in *E. coli* isolates collected around Berlenga Island (Portugal), comparing water isolates with those from human sewage and ~~seagull~~ yellow-legged gull, (*Larus michahellis*) feces at the same sites. Broad presence of AR was confirmed by base pair sequencing of *E. coli* genes. The data documented that sea water clearly is an AR reservoir, in that multi-resistance was high in coastal waters isolates. Sea gull-sourced water contamination was more contributory than sewage-sourced contamination, underscoring the avian role in multi-faceted ecological influence on dissemination of AR *E. coli* (Alves et al 2014). Additional complexity is introduced by the fact that ~~sea~~ gull~~s~~ (*Larus* spp.) are known to associate with human garbage, waste water effluent, and beaches, suggesting that species-environment-species sequential relationships may be diverse and could involve both biological and mechanical vectoring (Sjolund et al 2008).

Fecal *E. coli* isolates from 3 closely-related gull species, the lesser black-backed gull (*Larus fuscus*), herring gull (*Larus argentatus),* and yellow-legged gull (*Larus michaellis*), were evaluated for AR (Stedt et al 2014). Specimens were collected in Denmark, England, Ireland, Latvia, Netherlands, Poland, Portugal, Spain, and Sweden, yielding 3158 samples and producing 2210 *E. coli* isolates. AR was noted in 31.5% of isolates, with 19% displaying multiple AR. Lowest AR frequency occurred in Denmark and Ireland, with highest AR in Spain and Latvia. AR in *E. coli* strains clearly occurred beyond clinical and agricultural environments. Flock foraging in fields, ~~dumps~~ open municipal land fills, harbors, and generally near humans, likely contributed to AR dissemination. Given the long-distance migration habits of many gulls, single flocks can be composed of individuals from widely varying geography, further supporting wide AR dissemination by migrating birds (Stedt et al 2014).

A study using metagenomic methods revealed that fecal isolates from four herring gulls at Shoals Marine Laboratory, Appledore Island, Maine (USA) were carrying AR genes that often are have been found in specimens from humans and domestic animals, again strongly suggesting transmission of AR genes from terrestrial species to migrating birds (Martiny et al 2011).

Do marine predators other than seals carry organisms with AR genes?

Schaefer and colleagues (2009) evaluated 909 culture swabs from blowhole, gastric fluid, and feces of 171 dolphins in multiple collection areas. Human and animal pathogens were observed, along with site-related variation. AR was identified in over 50% of isolates, not surprising in that dolphins regularly are exposed to human waste and agriculture run-off.

Multisite cloacal sampling of marine predatory fish also revealed AR bacteria, with MDR noted at several sampling sites. Fish species may represent yet another aquatic reservoir for AR, and the authors observed that long-lived fish may exert quantitative influence on AR dissemination (Blackburn et al 2010).

Do we know the future of AR?

Dissemination of AR DNA by both pathogenic and non-pathogenic bacteria, vectored in various ways, has been mechanistic for establishing the ubiquitous global presence of AR in terrestrial and aquatic environments (Beatson & Walker 2014). If the genetic context of AR DNA within the cell has importance in this process, then spatial and biochemical intracellular features should be evaluated by analytical methods such as long-read genome sequencing of bacteria (Beatson & Walker 2014). Beatson and Walker point out that knowing more about number, position, transfer, epidemiology, and evolution of AR genes is an avenue for elucidative research that cannot be done with the same short-read technology that is used presently for strain identification and establishing sources of individual contamination events (Beatson & Walker 2014).

Another study using long-read analytical methods showed that AR genes can be carried by plasmids that are different, numerous, and multiple within bacteria (Conlan et al 2014). Without whole-plasmid sequencing, the complete information complement that is needed to fully understand transfer and reservoir processes will not be available and phylogenetic or epidemiologic inferences may be compromised (Conlan et al 2014).

Our database indicated that most abscesses in seals arise from conspecific bite wounds, but microbiological examination shows that the abscesses largely reflect environment-sourced microorganisms rather than “indigenous” species. These observations suggest that the biology of the water-oral interface of marine mammals, and the resulting outcome of bacterial competition during abscess development, are insufficiently understood.

As long-distance migratory pathways habits of many birds and marine mammals intersect more frequently with moving humans and other animals, opportunities for new AR DNA exchanges among intermingling bacterial are likely to increase. The effects of agricultural practices; animal and human migrations; worsening marine pollution; geopolitical turmoil; and climate change, are ecologically disruptive. The microbiological consequences of combinations of these disruptive events are not yet fully understood, but unfolding AR data indicate that elucidation is very critical.

Will continuing production of new antibiotics be effective or ultimately counterproductive? Despite the huge expense and logistical problems, is it time to entirely re-think concentration and confinement rearing of food-producing animals, world-wide? For species preservation, including our own, do we finally need to address environmental sanitation on a world-wide scale? Existing microbiological data suggest that new human pathways will be needed in all of these areas. We do not yet know where and when the point of species collapse will be reached.

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Table 1. Antibiotic classes and representative compounds

Penicillins: amoxicillin, amoxicillin-clavulanic acid, piperacillin
Cepahlosporins: ceftiaxine, ceftiofur, cephalexin
Aminoglycosides: amikacin, gentamycin, tobramycin
Flouroquinolones: ciprofloxacin, enrofloxacin
Tetracyclines: tetracycline
Phenicols: chloramphenicol
Folate pathway inhibitor: trimethoprim/sulfa

Figure 1.

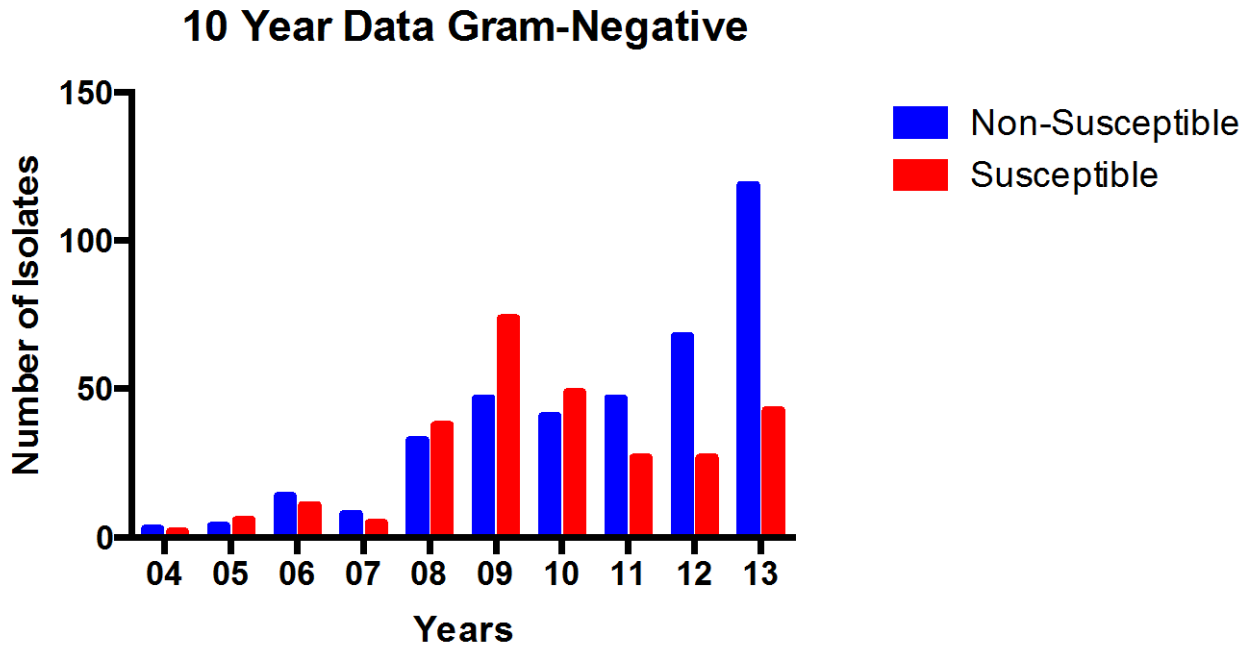

Figure 2.

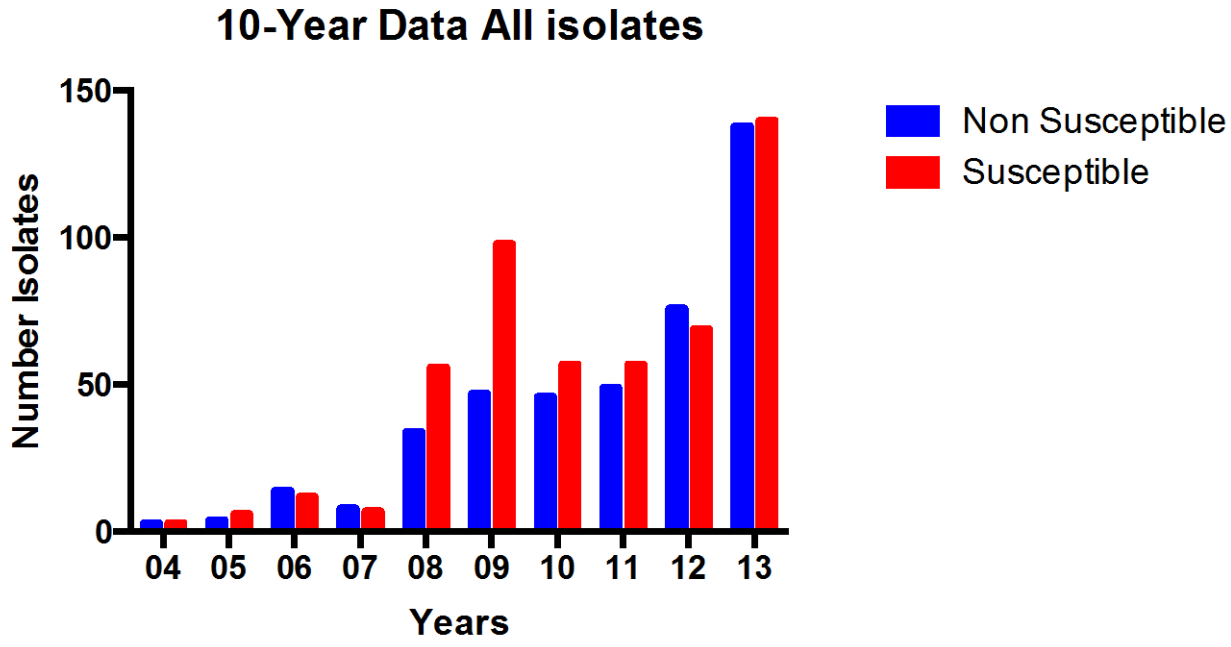

Figure 3.