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Review on Pre-clinical Antimicrobial Assay

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ABSTRACT

Pre-clinical antimicrobial testing is one costly step in antimicrobial drugs development. Costly effective methods in performing the in vitro and in vivo assay as part of pre-clinical stage is critical. We reviewed the current development of this stage. We found that standardization of agar diffusion techniques and measurement of minimal inhibitory concentrations in broth dilution methods serve as the primary reference for in vitro antimicrobial testing. In vivo, moral issues, ethics, costs, and the correlation of using animal models with human physiological conditions enforce us to seek alternative systems or animal models. Organ-on-a-Chip (OC) emerges as an ethically sound alternative system, yet in terms of cost and simulation of physiological conditions, there is still much progress to be made. Fruit fly (*Drosophila melanogaster*) and waxmoth (*Galleria mellonella*) are currently the main alternative animal models that are more affordable, simple, and ethically sound compared to worms, silkworms, mice, and primates. Artemia spp. and Hydractinia spp. have the potential to become new alternative animal models in simulating microbial infections and the efficacies of the antimicrobial that fight against it in the future.

MICROBES KILL HUMAN

Narrated by Abu Hurairah from the Prophet Muhammad ﷺ:

" لاَ عَدْوَى وَلاَ طِيَرَةَ وَلاَ هَامَةَ وَلاَ صَفَرَ، وَفِرَّ مِنَ الْمَجْذُومِ كَمَا تَفِرُّ مِنَ الأَسَدِ ".

There is no contagious disease, no superstition in birds, no ghoul, and no evil omen in the month of Safar. But flee from the leper as you would flee from a lion" (al-Bukhari, 846). This hadith seemingly inspired Ibn Sina, in his Qanun At-Thib, which was translated into Latin and then into English as the Canon of Medicine, to state: "*Body secretions of a host organism (e.g., human being) are contaminated by tainted foreign organisms that are not visible by naked eye before the infection occurs*" (Colgan, 2010). In principle, he stated that in the body secretion from a corpse infected with a contagious disease, there must be a foreign body that cannot be seen by the naked eye (microorganisms) which initially caused the disease. Leprosy, a disease that the Prophet ordered us to flee from, was the first disease definitively found to be caused by bacteria, *Mycobacterium leprae*, by a Norwegian doctor in 1880 shortly after Robert Koch introduced the method for staining bacterial samples (Vogelsang, 1963).

A 2020 report by the World Health Organization (WHO) stated that microorganisms such as bacteria, fungi, protozoa, and molds infect humans in the lower respiratory tract and in infants are among the top two leading causes of human death from the five leading causes of death in 2019. In the same report, ischemic heart disease listed as the number one cause of death and killed about 9 million people in 2019 (WHO, 2020). However, the Global Burden of Disease (GBD), a global organization founded by WHO in the 90s but now fully funded by the Bill & Melinda Gates Foundation, reported that bacteria killed more than 11 million human in 2019 (GBD 2019 Antimicrobial Resistance

Collaborators, 2022). This report only included data on deaths caused by bacteria. If data on other microorganisms were included, then deaths from fungi and molds such as *Candida albicans* and *Malassezia furfur*, as well as deaths from protozoa such as *Plasmodium falciparum* (causing malaria) and *Trichomonas vaginalis* (causing vaginosis), would inflate human death data caused by microorganisms.

TESTING DRUG CANDIDATES OF ANTIMICROBIALS

Narrated by Abu Hurairah from the Prophet Muhammad ﷺ:

مَا أَنْزَلَ اللَّهُ دَاءً إِلاَّ أَنْزَلَ لَهُ شِفَاءً فَإِذَا أُصِيبَ دَوَاءُ الدَّاءِ بَرَأَ بِإِذْنِ اللَّهِ عَزَّ وَجَلَّ عَلِمَهُ مَنْ عَلِمَهُ وَجَهِلَهُ مَنْ جَهِلَهُ

Allah has not sent down a disease without sending down a cure for it. If the correct remedy is applied to the disease, it will be cured by the permission of Allah, the Exalted and Glorious. The knowledgeable know it, and the ignorant do not." (Hanbal, 842; al-Bukhari, 846; Muslim, 875). Therefore, it becomes the duty for Muslims with knowledge to seek remedies as an effort to cure various infectious diseases caused by microorganisms.

The efficacy of drug substances in killing or inhibiting the growth of microorganisms (antimicrobials) needs full attention from researchers to reduce human mortality due to infectious diseases in the future. Furthermore, bacteria can adapt by acquiring resistance to antimicrobial drugs (WHO, 2023; Liu, et al., 2016). Unfortunately, the livestock and aquaculture industries, which are sources of human food and animal protein, often use antimicrobial substances wastefully, which actually accelerates the bacteria's acquisition of drug resistance (Schar, et al., 2021; Mulchandani, Wang, Gilbert, & Van Boeckel, 2023). This urgently calls for global efforts in finding new-generation antimicrobial drug candidates. One of the stages in the discovery of new antimicrobial drugs is by testing various drug candidates against the growth of various pathogenic bacteria, which we call Antimicrobial Assay.

Preclinically, drug candidates (including antimicrobial drug candidates) are tested *in vitro* and *in vivo* before clinical trials. A critical and elegant review of *in vitro* antimicrobial testing has been previously discussed (Eloff, 2019). In this discussion, we present it briefly.

IN VITRO ANTIMICROBIAL ASSAY

In vitro, the most popular test is diffusing the active compound on agar media that has been/is being grown with microorganisms, known as the agar diffusion method. The type of agar media used varies depending on the microorganism being tested. There are two ways to diffuse the active compound: (1) making a hole in the agar media and placing the active compound in the hole to diffuse, and (2) soaking or dropping the active compound onto a paper disc and then placing it on the agar media. An area around the diffusion center (hole/disc) will form a zone where microbes do not grow, called the inhibition zone. The diameter/area of the inhibition zone that forms should be directly proportional to the concentration of the active compound being diffused and significantly larger than the inhibition zone produced by the solvent (Hewitt & Vincent, 1989).

Research results based on the agar diffusion method often cannot be well replicated by different researchers because there are many factors that must be controlled when using this method. These factors include agar density, bacterial inoculum density, concentration/volume of the active compound used, media composition, size of the hole/disc, incubation time, and incubation temperature. Although these factors are generally known by researchers, it is often practically difficult to ensure consistency. Therefore, strict guidelines for its clinical use to track antibiotic-resistant bacteria have been formally established by standardization bodies such as those in the United States and the European Union and have often been adopted in Southeast Asia (Cusack, et al., 2019).

Efforts to control the outcomes obtained from the agar diffusion method become more challenging if the active compound being tested is not a pure active compound, but rather an extract of a plant using various types of polar, semi-polar, and non-polar solvents. The difference in polarity between the active compound being tested and the positive control compound, combined with the influence of the polarity of the agar medium, makes the direct relationship between the diameter of the inhibition zone and the efficacy of the active

compound as a potential antimicrobial drug less certain. Therefore, this method is only recommended for the initial screening of compounds that have potential antimicrobial properties, but the ability to inhibit microbes must be confirmed with other methods (Klancnik, Piskernik, Jersek, & Mozina, 2010; Eloff, 2019).

The broth and agar dilution methods are the same in the process of diluting the active compound to be tested but differ in the testing process against the target microbes. In the agar dilution method, testing against microbial growth occurs on solid agar media, while in the broth dilution method, the inhibitory effect is observed against microbial growth in broth media. Test results show that the minimum inhibitory concentrations (MIC) found with these two methods are the same if the tested microorganisms are Gram-positive bacteria, but significantly different if the tested microorganisms are Gram-negative bacteria, with the broth dilution method revealing a smaller MIC (Klancnik, Piskernik, Jersek, & Mozina, 2010).

The methods for observing microbial inhibition in the broth dilution method vary and can be simply distinguished based on the scale of the testing. On a macro scale, turbidity observation can be done visually or with instruments. On a micro scale, visual observation of turbidity becomes difficult, so the broth is stained with a colored indicator (i.e. tetrazolium based staining) that exhibits signs of life (Klancnik, Piskernik, Jersek, & Mozina, 2010; Lall, Henley-Smith, De Canha, Oosthuizen, & Berrington, 2013). Differences in the scale of dilution and observation (micro and macro) display insignificant differences thus similar conclusions in test results for diverse compounds and many bacteria tested. This indicates that the broth dilution method is indeed the preferred method to determine the efficacy of a compound as a potential antimicrobial drug (Klancnik, Piskernik, Jersek, & Mozina, 2010; Eloff, 2019).

In fact, EUCAST, the European body for standardizing methods of testing bacterial resistance to antibiotics in clinical settings, mandates the use of either the broth or agar dilution method for complex bacterial cases. (Cusack, et al., 2019).

BEYOND *IN VITRO* TEST

Among the many molecules or plant extracts proven effective as antimicrobials *in vitro*, often they are not effective when tested *in vivo*, let alone clinically. *In vivo* testing is necessary to determine whether antimicrobial candidates remain effective when faced with the physiological conditions of animals, such as pH, temperature, surrounding chemicals, and the potential presence of natural enzymes in animal and human bodies that may inhibit the efficacy of the candidate drug. Additionally, *in vivo* tests also assess the toxicity of the candidate drug for animals and humans in general. The balance between efficacy and toxicity is measured at various dosages, so that an effective antimicrobial dose that is not toxic to animal organs can be identified. This dosage is expected to predict a similar balance in humans during the clinical trial phase.

When considering the *in vivo* testing, researchers typically think of testing the candidate drug on mice. The next choices usually include rats, rabbits, primates (non-human), and sometimes zebrafish (*Danio rerio*). These model animals are commonly used to mimic physiological events that are strongly related to human physiological processes. Reviews on the use of these animals for antimicrobial testing have been widely published (Zak, O'Reilly, & , 1991; Singh & Gupta, 2018; Jensen, 2020).

Thousands of drugs have passed the dosage balance test between efficacy and toxicity on various animal models mentioned above, but upon systematic review, these dosages have often failed to predict the expected dosage in humans. This failure incurs extremely high costs. These costs are not only for the expenses of experimentation and animal maintenance but also for the time required because of the prolonged period before a drug can be approved by regulatory bodies (Van Norman, Limitations of Animal Studies for Predicting Toxicity in Clinical Trials, 2019). If ultimately the toxicity tests on animal models are only a rapid screening for acutely dangerous drugs, but the drugs that passed the screening still need to be clinically proven. Therefore, efforts to accelerate and simplify the cost of *in vivo* testing become imperative.

From an ethical perspective, infecting animal models with microorganisms and then killing

them to examine their physiological conditions raises another question that needs to be addressed. Is it appropriate for scientists to kill hundreds of animal models to test the efficacy and toxicity of a single drug?

REPLACING *IN VIVO* TEST

One of the philosophical sources of Western ethics, Immanuel Kant, regarded humans as **rational** beings with no obligation towards irrational beings like animals (Birch, 2020). Thus, there is no moral obligation for humans to feel guilty about using or even mistreating animals for human purposes. In Islamic ethics, Allah says:

هُوَ ٱلَّذِى خَلَقَ لَكُم مَّا فِى ٱلْأَرْضِ جَمِيعًا

He who created for you all that is in the earth" (Al-Baqarah 2:29), indicating the permissibility of using what is on the earth - including animals - for human benefit. However, the Prophet Muhammad ﷺ said:

إِنَّ اللَّهَ كَتَبَ الإِحْسَانَ عَلَى كُلِّ شَىْءٍ فَإِذَا قَتَلْتُمْ فَأَحْسِنُوا الْقِتْلَةَ وَإِذَا ذَبَحْتُمْ فَأَحْسِنُوا الذِّبْحَ وَلْيُحِدَّ أَحَدُكُمْ شَفْرَتَهُ فَلْيُرِحْ ذَبِيحَتَهُ

Indeed, Allah has prescribed excellence in all things. So if you kill, kill well; and if you slaughter, slaughter well. Let each one of you sharpen his blade and let him spare suffering to the animal he slaughters." (Muslim, 875). This hadith emphasizes the importance of treating animals well, even when killing them. The Prophet ﷺ also prohibited intentionally causing harm to animals.

ذِّبَتِ امْرَأَةٌ فِي هِرَّةٍ حَبَسَتْهَا، حَتَّى مَاتَتْ جُوعًا

A woman was punished in Hell because of a cat which she had confined until it died of hunger." (al-Bukhari, 846). Furthermore, Imam Muslim narrates more than six hadiths from the Prophet ﷺ condemning those who use animals for target practice or kill animals without a purpose for benefit in a specific chapter titled "Prohibition of Torturing Animals" (Muslim, 875). Thus, in Islam, there is a balance between the permissibility of humans to 'utilize' animals and the prohibition of torturing them.

A simple solution in this regard is to replace animal models with other methods to test the dosage balance between efficacy and toxicity of a drug. Other methods currently known to us are at least divided into three: *In silico*, Organ-on-a-Chip (OC), and still *in vivo* but using alternative animal models. The first two have been extensively discussed by Dr. Gail (Van Norman, Limitations of Animal Studies for Predicting Toxicity in Clinical Trials Part 2, 2020). In summary, he states that despite significant efforts to develop computer modeling (*in silico*) to closely resemble human conditions, the possibility of using it to replace *in vivo* testing is still far-fetched. Therefore, he advocates for the OC approach as a replacement for experiments using animal models.

ORGAN-ON-A-CHIP (OC)

In principle, Organ-on-a-Chip (OC) is an effort to cultivate specific human tissue cells in a particular three-dimensional structure that is maintained and controlled in a microfluidic manner, meaning a system for controlling the flow of fluids at the microelectronic level (hence called Chip). This system tries to mimic the three-dimensional space and function of bodily organs better than just cultivating one specific human cells.

The OC method for studying *Shigella* spp. infection in intestinal conditions has been published (Grassart, et al., 2019). In fact, intestinal OC has been used to resemble human microbiome conditions containing more than 200 species of microorganisms (Jalili-Firoozinezhad, et al., 2019). Recent developments even show conditions very similar to the real human intestine *in vivo* (Nikolaev, et al., 2020). Similarly, OC modeling of *Mycobacterium tuberculosis* infection in the lungs has been conducted (Thacker, et al., 2020). These intestinal and lung OC models serve as examples of how microbial interactions with intestinal and lung functional conditions can be simulated without the use of animal models. Unfortunately, although this method can simulate the efficacy of candidate drugs in intestinal or lung functional conditions, it cannot simulate drug toxicity in the same way as *in vivo* testing, as toxicity of a compound more commonly occurs in the liver organ, rather than in target organs like the intestine or lungs. Therefore, researchers should use at least two models: OC specific to the drug's target organ, as well as OC for testing drug toxicity in organs like the liver.

Liver OC has also been developed (Jang, et al., 2019). However, its modeling is often directed towards alcohol toxicity and liver disease. The level of cell diversity in the liver organ also

makes liver OC modeling, which can only include 3-5 cell types, difficult to compare with *in vivo* liver organs (Moradi, et al., 2020). On the other hand, one of the initial goals of replacing animal models was to reduce costs, speed up screening time, and simplify the preclinical testing process. OC technology demands the use of more than one OC system; one OC system (like lung and intestine) for testing drug efficacy, and one OC system (especially for the liver) for testing drug toxicity. Unfortunately, toxicity not only applies to the liver organ but can also frequently occur in other organs such as nerves and kidneys. Therefore, the use of OC as a replacement for animal models in testing candidate drugs becomes more complicated. Efforts to simulate more than one organ in one OC system have also begun, but they are still a distance away from the expectation of replacing *in vivo* testing (Jalili-Firoozinezhad, Miranda, & Cabral, Modeling Human Body on Microfluidic Chips, 2021).

ANIMAL MODELS REPLACEMENT

Replacing *in vivo* animal models with alternative animal models fundamentally does not eliminate ethical issues. There are still animals being tested, which means they are subjected to suffering (infected with pathogens to induce illness) and then killed for organ observation. However, alternative animal models can simplify the *in vivo* testing process, thus reducing costs and the time required for preclinical stages. Although in Islam, there seems to be no distinction between large animal models like rabbits, mice, and primates and smaller organisms like worms and flies. In terms of modern Western cultural philosophical ethics, these two types of animals are considered significantly different. Therefore, replacing mice and rats, which have a central nervous system and are costly, with animal models that have simpler nervous systems and lower costs such as flies (*Drosophila melanogaster*) and worms (*Caenorhabditis elegans*) becomes an important way to address ethical issues for researchers in Western culture (Freires, Sardi, de Castro, & Rosalen, 2017; Cheluvappa, Scowen, & Eri, 2017; Kaito, Murakami, & Furuta, 2020).

The fruit fly *Drosophila* has become an important animal model for understanding the innate immune system in animals in detail (Liegeois & Ferrandon, 2022). The success rate of human infection modeling by *Drosophila* is high and diverse, not only for common pathogenic bacterial microorganisms such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, but also the Zika virus, *Candida albicans* fungus, and unique bacteria like *Listeria monocytogenes* (Dionne, Ghori, & Schneider, 2003; Mansfield, Dionne, Schneider, & Freitag, 2003; Davis, et al., 2011; Yuan, et al., 2018; Toure, Herrmann, Szuplewski, & Girard-Misguich, 2023).

The worm, *Caenorhabditis elegans*, as an animal model has also been widely recognized in modeling various biological conditions. However, as an animal model for infectious disease caused by microorganisms, the use of *C. elegans* is still more limited compared to *Drosophila*. To our knowledge, although many bacteria have been tested for infecting this worm, only about five species of microorganisms (*E. coli*, *P. aeruginosa*, *Bacillus thuringiensis*, *Enterococcus faecium*, and *Salmonella* spp.) have consistently simulated infection in this animal model and are still being further researched to this day (Kim & Flavell, 2020). It may be physically difficult to inject bacteria directly into the body of *C. elegans*, which is very small, whereas researchers who use *C. elegans* as an animal model rarely use microinjection systems. Therefore, infecting *C. elegans* typically occurs orally because these worms naturally consume bacteria. In contrast, researchers who use *Drosophila* as an animal model are accustomed to working with microinjection systems, so the relatively larger size of *Drosophila* larvae may seem very large compared to the body of *C. elegans* to be injected with microorganisms under a microscope using microinjection (Kaito, Murakami, & Furuta, 2020).

Comparing the two animal models above, it is clear that *Drosophila* is superior for use in disease infection modeling and testing candidate drugs as therapies. However, infected flies pose a risk of escaping from the laboratory into the environment and infecting other flies that interact with human food. Moreover, although not as expensive as mice, anesthetizing and infecting *Drosophila* requires expertise and unique equipment that also require funding (Kaito, Murakami, & Furuta, 2020).

There are actually several other animals (i.e. crickets, silkworms, and hornworms) that have been attempted to be used as animal models for microorganism infection. However, these efforts have not been widely accepted and are limited to

a few laboratories (Kaito, Murakami, & Furuta, 2020). In contrast, the wax moth (*Galleria mellonella*) has recently become a celebrity in animal model for studying the process of microorganism infection (Asai, Li, Newton, Robertson, & Langford, 2023).

The wax moth larvae are relatively large and do not require a microscope to observe and then inject pathogenic bacteria. The progression of the disease caused by microbial infection can be easily observed with the change in color of the larvae from yellow to brown and even deep black. Moreover, maintaining wax moth larvae is relatively easy. Coupled with the possibility of raising wax moth larvae at 37°C, the temperature at which human pathogenic bacteria grow, places the wax moth in a very strategic position as an animal model, as all other alternative animal models we have discussed here so far cannot live normally at 37°C (Asai, Li, Newton, Robertson, & Langford, 2023). Using the keywords "*Galleria mellonella bacterial infection*" in Google Scholar search engine at the time of this review written, yielded 3,820 articles since 2023, and more than 11,000 articles since 2020, but only ~4,000 articles before 2010. This indicates an explosion of research using waxmoths as an animal model for bacterial infections after 2010, although this research has been around since 1987, even referring to earlier studies in 1963 (Morton, Dunphy, & Chadwick, 1987; Asai, Li, Newton, Robertson, & Langford, 2023). The waxmoth is naturally a pest insect in beekeeping, which has also been extensively studied in Indonesia (Vindri, 2018; Raharjo, 2021).

POTENTIAL ANIMAL MODELS

Considering the goal of using animal models to assess the balance between the efficacy and toxicity of candidate drugs, animal models commonly used for toxicity testing that can be infected by microorganisms should also be considered as potential animal models, similar to wax moths. There are two well-established animal models for toxicity testing known to be susceptible to microbial infection, namely *Artemia* spp. and *Hydractinia* spp. The downside of *Artemia* and *Hydractinia* is that they are both marine animals, so observing the process of microbial infection in marine animals compared to terrestrial animals may not be entirely comparable.

However, *Artemia* serves as a food source for fish and other aquatic animals and is widely available commercially. Furthermore, *Artemia* is a well-established animal model for toxicity testing (Libralato, Prato, Migliore, Cicero, & Manfra, 2016). Studies examining the interaction between *Artemia* and bacterial infection have also been conducted (Zheng, et al., 2011; Zhang, et al., 2018). Efforts to infect *Artemia* with a specific human microorganism and then test the efficacy of potential antimicrobial drugs afterwards present an interesting and challenging endeavor for the future. Nevertheless, *Artemia* have been used mostly to model host-pathogen relationship foraquaculture infections (Baruah, Phong, Norouzitallab, Defoirdt, & Bossier, 2015; Roy, Baruah, Bossier, Vanrompay, & Norouzitallab, 2022). However, one report the usage of *Artemia* for humans' *Pseudomonas aeruginosa* infections (Lee, Kim, Li, & Lee, 2014).

Similarly, *Hydractinia* spp. has been used for toxicity testing of various compounds (Chicu, Schannen, Putz, & Simu, 2016; Chicu S. A., 2019). The interaction between *Hydractinia* spp. and bacteria has also been extensively studied (Guo, et al., 2017; Guo, Rischer, Wastermann, & Beemelmanns, 2021). Interestingly, both studies (both toxicity testing and bacterial interaction) were conducted in the context of larval metamorphosis, with the observation period being less than 48 hours. This is the main advantage of *Hydractinia* spp. compared to other animal models. However, as in the case of *Drosophila*, equipment for microscale injection is required to establish research on microbial infection in *Hydractinia* larvae and to observe its effects on the metamorphosis process, which serves as a model for the general physiological conditions of animals. Moreover, cancer can induced in *Hydractinia* (Cathriona, et al., 2011), allowing for a possible microbes infections-cancer modelling, which draw strong attention recently (Dzutsev, et al., 2017; Galloway-Pena, Iliev, & McAllister, 2024). Nonetheless, despite the fast available information on their innate immune system (Zarate-Potes, Ocampo, & Cadavid, 2019), testing *Hydractinia* with an actual human microbial pathogen is a necessary step to bring them into the list of animal model for infectious diseases.

CONCLUSION

In vitro test of antimicrobial drug candidates, apart from agar diffusion, has to be performed also with broth dilution method. *In vivo*, Fruit flies (*Drosophila melanogaster*) and waxmoths (*Galleria mellonella*) are currently the two main and popular model organisms for simulating

microbial infections and assessing the efficacy and toxicity of active compounds used to combat these infections. Both can be easily and cheaply bred in large numbers and mimic the general physiological conditions of animals quite closely to the human innate immune system.

In the future, *Artemia* spp. and *Hydractinia* spp. may have the potential to become new, relatively simpler animal model replacements compared to the two insects mentioned above for simulating microbial infections and their treatments. Moreover, once OC can simulate two-three organ simultaneously in the future, it will become a serious contender for *in vivo* assay.

AUTHORS' CONTRIBUTIONS

Febrimarsa provided conception; study; conducted data collection; analysis & interpretation; critical revision; article drafting and approved final version.

CONFLICT OF INTERESTS

The author declare that there is no conflict of interests regarding the publication of this article.

ETHICAL CONSIDERATION

Ethical issues (including plagiarism, data fabrication, double publication, etc) have been completely observed by the authors.

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