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Aeromonas hydrophila as an environmental indicator to detect TiLV-infected tilapia under coinfection threat

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ABSTRACT

As an emerging infectious agent threatening freshwater ecosystems and tilapia aquaculture, tilapia lake virus (TiLV) has expanded its geographical range over last five years. Seeking a way to alleviate loss of tilapia production caused by TiLV is an urgent need for commercial aquaculture. Recently, *Aeromonas hydrophila* (AH) was found to be concurrent with TiLV causing serious issues. However, our understanding of how coinfection of AH and TiLV impact on disease transmission dynamics remains limited, particularly in real farming ecosystems, where difficulty of direct experimentation makes inference challenging. Here we applied epidemiological models to fit mortality data of tilapia single- or co-infected with TiLV and AH to estimate key transmission parameters that govern virus-bacteria interactions among infection states. Dose–response analysis was performed to assess the effects of AH concentration on TiLV disease transmissibility and mortality. Results showed that basic reproduction number (R_0) for TiLV transmission in population that infected with TiLV followed by higher load of AH could be reached ~11. Our dose-response model reveals that AH concentration in fish pond can be a key environmental indicator to drive epidemiology-based measure as well as to assess exacerbating mortality risk of TiLV-infected tilapia under coinfection. The model may therefore be useful for guiding the future development of control measures from an epidemiological perspective to mitigate the impact of TiLV disease on tilapia.

1. Introduction

As an emerging infectious agent that can cause significant mortality of up to 90%, tilapia lake virus (TiLV) has aroused a global-level concern of food security (Dong et al., 2017a; FAO, 2017; OIE, 2018). After firstly confirming TiLV by Eyngor et al. (2014) (OIE, 2018), several studies have dedicated to evidence the infection of TiLV in tilapia (Aich et al., 2021) and to rapidly detect the virus (Kembou Tsofack et al., 2017; Dong et al., 2017b; Liamnimitr et al., 2018). In addition to horizontal transmission, TiLV was recently found to be transmitted vertically from broodstock to their reproductive organs and fertilized eggs (Dong et al., 2020). The distribution of countries reporting TiLV cases has covered Africa, Asia, and Americas (Thawornwattana et al., 2020; USDA, 2019a).

Undoubtedly, the aforementioned studies mainly concerned only on pathogen TiLV. In fact, however, coinfections commonly occur when hosts are infected by two or more different pathogens (Kotob et al., 2016). Generally, interactions between pathogens cooccurring within a single host could have significant effects on infection outcomes, covering from severity of clinical disease in individual hosts to disease spread rate across populations (Kotob et al., 2016). Thus, understanding the consequences of pathogen interactions during coinfection is essential for effective disease control and management.

As coinfection of aquatic animals has rarely received attention (Kotob et al., 2016), information on coinfection of TiLV and other pathogens is even hardly available. It was reported that higher mortalities found in TiLV-positive tilapia were attributable to secondary infection of bacteria (*Flavobacterium*, *Aeromonas*, and *Streptococcus*) and parasites (*Gyrodactylus* and *Dactylogyrus*) (Surachetpong et al., 2017). Among these pathogens, *Aeromonas* species such as *A. enteropelogenes*, *A. hydrophila*, *A. veronii*, and *A. ichthiosmia* were also detected in TiLV-infected tilapia (Amal et al., 2018; Nicholson et al., 2017). As a bacterium widely distributed in fresh water, bottom sediments, and intestinal tract of fish, *A. hydrophila* causes a haemorrhagic septicaemia which is one of the most significant diseases occurring in cultured freshwater fish (CABI, 2019), with no exception for tilapia. Moreover, post-coinfection of TiLV and *A. hydrophila* was found to worsen disease severity in tilapia, motivating the need for developing the strategies to

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mitigate the coinfection risk (Nicholson et al., 2020).

It is suggested that tilapia producers should be aware of the effect of coinfections on the course of disease (Abdel-Latif et al., 2020). Generally, the population-level mathematical models were commonly used in aquaculture production to get insight into the spread of infectious diseases (Sitjà-Bobadilla and Oidtmann, 2017). These models are usually designated as SI-, SIS-, SIR-, and SEIR-based models by reasonably dividing population into susceptible (S), exposed (E), infected (I), and recovered/removed (R) states in which a homogeneously mixing population is assumed (Ben-Horin et al., 2018; Hoover et al., 2019; Karvonen et al., 2019; Krkošek, 2010; Kumar et al., 2017; Liao et al., 2006; Lotz and Soto, 2002; Murray, 2013; Ogut et al., 2005; Salama and Murray, 2011; Werkman et al., 2011). Most of them, however, were developed for single infection, apart from that developed by Karvonen et al. (2019) in that the effect of multiple infections on virulence of pathogens–host interactions was well-explored.

Even though there are studies tackling the topics of the coinfection dynamics by mathematical modeling, issues are popularly focused on human diseases (Aldila and Agustin, 2018; Barley et al., 2012; Nthiiri et al., 2015; Ogunmiloro, 2019; Osman and Makinde, 2018; Tilahun, 2019). However, application of the coinfection models for assessing the epidemic dynamics of fish diseases, to our knowledge, is limited. Taking account of the need of reducing the potential of worsening the disease due to coinfection and advantage of applying the disease transmission dynamic models, in the present study, we aimed at quantifying the effects of *A. hydrophila* on TiLV transmission by understanding the coexistence of TiLV and *A. hydrophila* in tilapia population to provide implications for disease management.

To this end, the purpose of this study is threefold: (1) to apply the single infection model for tilapia single-infected with TiLV or *A. hydrophila*, (2) to develop the coinfection model for tilapia coinfected with TiLV and *A. hydrophila*, and (3) to quantify the effect of *A. hydrophila* on TiLV transmission by constructing the dose–response relationships.

2. Materials and methods

2.1. Study data

In the present study, we used the literature-derived data to assess potential drivers of tilapia responses to post-coinfection of TiLV and *A. hydrophila*. Thankfully, Nicholson et al. (2020) have provided the most valuable cumulative mortality data on coinfection of TiLV and *A. hydrophila* in tilapia.

Briefly, red hybrid tilapia (*Oreochromis* spp.) were acquired from a local fish farm and then be acclimatized for 3 weeks. To reveal the effect of TiLV–*A. hydrophila* (TiLV–AH) coinfection on disease severity, 180 tilapia were assigned into six groups, and then were intraperitoneally (IP) injected with pathogens based on the following experimental conditions: (1) in the control group, fish were injected with sterile tissue culture medium (Fig. S1A); (2) in the single-infection experiments, fish were injected, respectively, with TiLV, low load of AH, and high load of AH assigned as groups V, LB, and HB (Fig. S1B); and (3) in the coinfection experiments, fish firstly infected with TiLV. After 3 days, fish injected with low and high loads of AH were assigned as groups V + LB and V + HB, respectively (Fig. S1C). In this experiment, low load of AH with 10^6 CFU fish^{-1}, high load of AH with 10^7 CFU fish^{-1}, and TiLV concentration of 10^4 TCID50 fish^{-1} were employed. Time-course cumulative mortality was observed in each group (Fig. S1D; Table S1). Mortality was not observed in the control group in which tilapia were not exposed to TiLV or AH.

Nicholson et al. (2020) also quantified AH concentration and viral load of TiLV in livers 5 days after TiLV exposure (i.e. 2 days after AH exposure) (Fig. S1D). The concentrations of AH in the groups V, V + LB, and V + HB were $7.50 \times 10^2 \pm 6.50 \times 10^2$ (Mean ± SD), $1.60 \times 10^3 \pm 7.2 \times 10^2$, and $1.4 \times 10^5 \pm 3.1 \times 10^4$ CFU mL^{-1}, respectively, indicating that the higher load of AH used in IP injection, the higher concentration of AH detected in livers. However, viral load of TiLV in the groups V, V + LB, and V + HB were similar, ranging from 3.2×10^2–6.7×10^3 copies per μg of fish tissue.

In this study, cumulative mortalities observed during the single- and co-infection experiments were applied to estimate epidemiological parameters for TiLV and AH transmissions by single-infection and coinfection models, respectively. The effects of TiLV–AH coinfection on tilapia responses were further assessed based on parameter estimates, quantitative load of AH together with mortality to implicate the disease mitigation strategies.

2.2. Single-infection model

The single-infection model was constructed to fit the cumulative mortalities observed during the single-infection experiments (groups V, LB, and HB) (Fig. S1). Tilapia population were divided into three subpopulations based on their health status of susceptible, infected, and mortality. Here we reasonably assumed that tilapia acquiring the diseases did not recover from infected state. The population dynamics of tilapia exposed only to TiLV or AH were described by a single-infection model that was modified based on the classical SIR model as (Eqs. (1)–(3); Fig. 1A),

$$\frac{dS(t)}{dt} = -k_i\delta(t-\tau_i) - \beta_i S(t)I(t) \quad (1)$$

$$\frac{dI(t)}{dt} = k_i\delta(t-\tau_i) + \beta_i S(t)I(t) - \alpha_i I(t) \quad (2)$$

$$\frac{dM(t)}{dt} = \alpha_i I(t) \quad (3)$$

Due to the limited time-course data on TiLV infection, we applied the disease transmission dynamic models to describe the experimental cumulative mortalities of tilapia infected with pathogens through IP injection. To model the transmission process resulted from exposure to pathogens existing in environment, we assumed that, initially, all the tilapia were susceptible (S) to pathogens without individuals infected. We used the term $k_i\delta(t-\tau_i)$ to capture impulse of initial infected population in our model where k_i is the generation of initial infected population per day (–), τ_i is the time for generating initial infected population (day), and $\delta(t-\tau_i)$ is the Dirac delta function defined as $\delta(t-\tau_i) = 0 t \neq \tau_i$, and $\int_{-\infty}^{\infty}\delta(t-\tau_i)dt = 1$representing the unit impulse function which is zero for all t except $t = \tau_i$. Here i could be V or B representing TiLV- or AH-specific parameter. Based on the single-infection model, susceptible tilapia exposed to TiLV could become infected state (I) with transmission rate β_V (day^{-1}) and die at mortality rate α_V (day^{-1}), whereas those exposed to AH could become infected state with transmission rate β_B (day^{-1}) and die at mortality rate α_B (day^{-1}). The dead tilapia were transferred into mortality state (M).

In the single-infection model, the basic reproduction number (R_0) is defined as the average number of new infections caused by a primary infected individual during the entire infectious period in a fully susceptible population (Anderson and May 1991). The R_0 could be calculated to play an important role in TiLV and AH transmissions denoted as β_V/α_V and β_B/α_B, respectively. As a measure of the potential for disease outbreaks, $R_0 < 1$ indicates that a disease will disappear over time, whereas $R_0 > 1$ means that there will be an epidemic (Anderson and May 1991; Keeling and Rohani, 2008).

2.3. Coinfection model

To describe the population dynamics of tilapia exposed to TiLV and AH, the coinfection model was constructed to fit the cumulative mortalities observed during the coinfection experiments (groups V + LB and V + HB) based on a two-disease epidemic model as (Eq. (4) – (8);

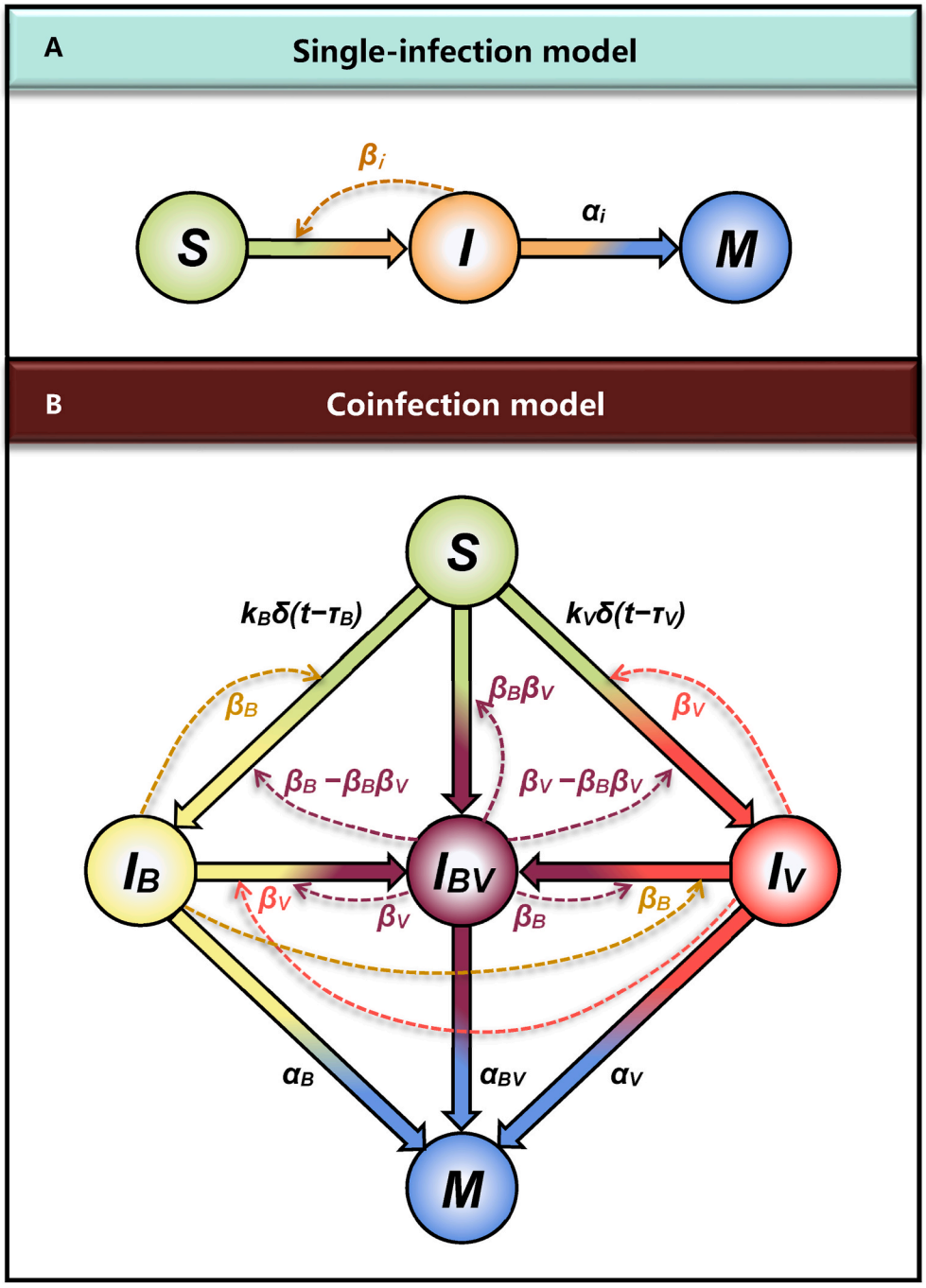

Fig. 1. Schematic describing the structure of (A) the single-infection and (B) the coinfection models used to describe the population dynamics of tilapia exposed to one of tilapia lake virus (TiLV) and *A. hydrophila* (AH) or both of them, respectively. The *S*, *I*, and *M* represent tilapia in susceptible, infected, and mortality states, respectively. The details regarding the epidemiological parameters were described in the text.

Fig. 1B) (Blyuss and Kyrychko, 2005; Gao et al., 2016),

$$\frac{dS(t)}{dt} = -k_B\delta(t-\tau_B) - k_V\delta(t-\tau_V) - [\beta_B I_B(t)S(t) + \beta_B(1-\beta_V)I_{BV}(t)S(t)] - [\beta_V I_V(t)S(t) + \beta_V(1-\beta_B)I_{BV}(t)S(t)] - \beta_B\beta_V I_{BV}(t)S(t) \quad (4)$$

$$\frac{dI_B(t)}{dt} = k_B\delta(t-\tau_B) + [\beta_B I_B(t)S(t) + \beta_B(1-\beta_V)I_{BV}(t)S(t)] - (\beta_V I_V(t)I_B(t) + \beta_V I_{BV}(t)I_B(t)) - \alpha_B I_B(t) \quad (5)$$

$$\frac{dI_V(t)}{dt} = k_V\delta(t-\tau_V) + [\beta_V I_V(t)S(t) + \beta_V(1-\beta_B)I_{BV}(t)S(t)] - (\beta_B I_B(t)I_V(t) + \beta_B I_{BV}(t)I_V(t)) - \alpha_V I_V(t) \quad (6)$$

$$\frac{dI_{BV}(t)}{dt} = (\beta_V I_V(t)I_B(t) + \beta_V I_{BV}(t)I_B(t)) + (\beta_B I_B(t)I_V(t) + \beta_B I_{BV}(t)I_V(t)) + \beta_B\beta_V I_{BV}(t)S(t) - \alpha_{BV} I_{BV}(t) \quad (7)$$

$$\frac{dM(t)}{dt} = \alpha_B I_B(t) + \alpha_V I_V(t) + \alpha_{BV} I_{BV}(t) \quad (8)$$

Briefly, tilapia population was divided into five states of susceptible (S), bacteria-infected (I_B), virus-infected (I_V), bacteria–virus-infected

(I_{BV}), and mortality (M) (Fig. 1B). While tilapia exposed to TiLV and AH simultaneously, they could be infected with one pathogen and then become AH- or TiLV-infected state (I_B and I_V) (Fig. 1B). Here tilapia take times τ_B and τ_V, respectively, to generate initial bacteria-infected (k_B) and virus-infected populations (k_V). Single-infected tilapia (bacteria- and virus-infected populations) not only can spread diseases by transmission rates (β_B and β_V, day^{-1}) but also can further be infected with the other pathogen and become coinfection state (I_{BV}) (Fig. 1B).

We assumed that susceptible, single-infection, and coinfection states have same contact rate; therefore, tilapia in states of I_B become I_{BV} by contacting I_V and I_{BV} with β_V, whereas those in state of I_V become I_{BV} by contacting I_B and I_{BV} with β_B. The transmission rates for susceptible become infected with AH, TiLV, and AH−TiLV after contacting tilapia in coinfection state (I_{BV}) are $\beta_B(1-\beta_V)$, $\beta_V(1-\beta_B)$, and $\beta_B\beta_V$, respectively (Fig. 1B). Tilapia in states of I_B, I_V, and I_{BV} would die at rates of α_B, α_V, and α_{BV} (day^{-1}), respectively, subsequently being transferred into mortality state (Fig. 1B). In the coinfection model, R_0s for TiLV-, AH-, and AH–TiLV-infections could be calculated, respectively, as β_V/α_V, β_B/α_B, and $\beta_V\beta_B/\alpha_{BV}$ (Gao et al., 2016).

2.4. Initial conditions of model fitting and model calibration

The present constructed single-infection and coinfection models were used to estimate epidemiological parameters by fitting the published cumulative mortality data (Table S1). In the fitting schemes, all the population was assumed to be susceptible at $t = 0$ and the ranges of τ_i and α_i were constrained based on observation of the mortality data in each scenario. While adopting the single-infection model to fit cumulative mortalities obtained from groups V, LB, and HB (Fig. S1B; Table S1), τs were constrained to be not earlier than 1 day and not be later than the day at which mortality was firstly observed. On the other hand, since the inverses of infectious periods are αs, infectious periods were limited to be not higher than the day at which mortality was firstly observed and not lower than one day, in order to constrain the ranges of αs.

For adopting the coinfection model to fit cumulative mortalities obtained from the groups V + LB and V + HB (Fig. S1C; Table S1), constrains for α_B and α_V were the same as those in single-infection fitting. However, there were differences in giving the limited ranges of τ_is between single-infection and coinfection scenarios. Since pathogen causing firstly-observed mortality was not confirmed, τ_B and τ_V were constrained to be not higher than the last day during observation. Moreover, in the coinfection experiments, tilapia were exposed to AH 3 days after TiLV exposure; as a result, lower bounds for τ_B and τ_V were 3 days and 1 day, respectively.

Here we adopted normalized root-mean square error (NRMSE) to assess performance of the single-infection and coinfection models based on the criteria: NRMSE values in the ranges of <10%, 10–20%, and 20–30% indicate that simulations are excellent, good, and fair, respectively, whereas > 30% is poor. NRMSE can be calculated as $\mathrm{NRMSE}(\%) = \sqrt{[\sum_{i=1}^{N_O}(P_i - O_i)^2]/N_O} \times (100\%/\overline{O})$ where N_O denotes the number of observations, O_i is the experimental data, P_i is the modeled result, and $\overline{O}$ is the mean of experimental data.

2.5. Dose–response analysis

In view of Nicholson et al. (2020), a dose-dependent fashion was found to be explicit on the relationships between TiLV–AH coinfection and disease worsening in tilapia population. Therefore, we tended to quantify the effects of AH on disease transmission and cumulative mortalities by applying the statistical models that were capable of well describing the very plausible relationships.

To assess the effect of AH on disease transmission, we constructed the relationships between exposure load of AH (L_B) and R_0 estimate. On the other hand, for the effects on cumulative mortalities: (i) the relationships between concentration of AH in livers at day 5 ($C_{BL,day5}$) and cumulative mortality at the same day (CM_{day5}) and (ii) the relationships between $C_{BL,day5}$ and increased cumulative mortality (CM_{INC}) observed between the same day and the day after an infectious period would be constructed based on concentrations of AH and epidemiological parameter estimates.

2.6. Modeling scheme

Model parameters can be estimated via least-square fitting by searching for a set of parameters that minimizes the sum of squared differences between the observed data and the corresponding model solution (Chowell, 2017). Hence, Berkeley Madonna 8.0.1 (Berkeley Madonna was developed by Robert Macey and George Oster of the University of California at Berkeley) was applied to estimate epidemiological parameters by adopting the single- and coinfection dynamic models to fit the time-course cumulative mortality data of tilapia. On the other hand, to construct the dose–response relationships, TableCurve 2D software (Version 5.01, AISN software, Mapleton, OR, USA) was employed to perform the model fitting and obtain the optimal statistic models describing estimated R_0 for TiLV transmission at different exposure load of AH and cumulative mortalities at different concentrations of AH in livers. Percentiles of 2.5th and 97.5th will be generated as the 95% confidence interval (CI).

The overall conceptual framework was demonstrated in Fig. 2 depicting (A) problem formulation, (B) mechanistic modeling processes, and (C) outbreak management for the impact of TiLV-AH coinfection on TiLV transmission.

3. Results

3.1. Parameter estimations: single infection

The single-infection model had optimal fits to cumulative mortalities observed in groups V (Fig. 3A), LB (Fig. 3B), and HB (Fig. 3C), respectively, with NRMSEs of 14.85, 14.81, and 20.43%. In group HB, it only took 2 days to generate initial infected population which was the earliest followed by groups V and LB (Table S2). The generation rates of initial infected population showed a subtle difference with values that were either 0.06 or 0.07 (Table S2). It should be noted that there was a significant difference among transmission rates in groups V (1.08 day^{-1}), LB (3.52×10^{-8} day^{-1}), and HB (1.23×10^{-4} day^{-1}) (Table S2). Results showed that TiLV was transmitted far much faster than AH (Table S2). Even though higher load of AH led to higher transmission rate, the value was much lower than that in group V (Table S2). Mortality was the highest in group V with 0.99, followed by 0.74 and 0.43 day^{-1} in groups LB and HB, respectively (Table S2).

3.2. Parameter estimations: coinfection

Results of fitting the coinfection models to cumulative mortalities of tilapia exposed to low and high loads of AH 3 days after TiLV exposure, showed excellent performances with NRMSEs of 5.93 and 8.89% for groups V + LB (Fig. 4A) and V + HB (Fig. 4B), respectively. The epidemiological parameters estimated in coinfection scenario could be classified into those for the transmission due to tilapia in TiLV-, AH-, and TiLV–AH-infected states (i.e. I_V, I_B, and I_{BV} states).

In group V + LB, initial populations infected with TiLV and AH were generated at rates of 0.08 and 0.10 after 5 and 2 days, respectively (Table S3). The transmission rates due to tilapia in TiLV-, AH-, and TiLV–AH-infected states were 0.90, 3.80×10^{-4}, and 3.40×10^{-4} day^{-1}, respectively, whereas mortality rates were 0.82, 0.91, and 0.48 day^{-1}, respectively.

In group V + HB, times for generating initial TiLV- and AH-infected tilapia with rates of 0.03 and 0.10 day^{-1} were 2 days and <1 day,

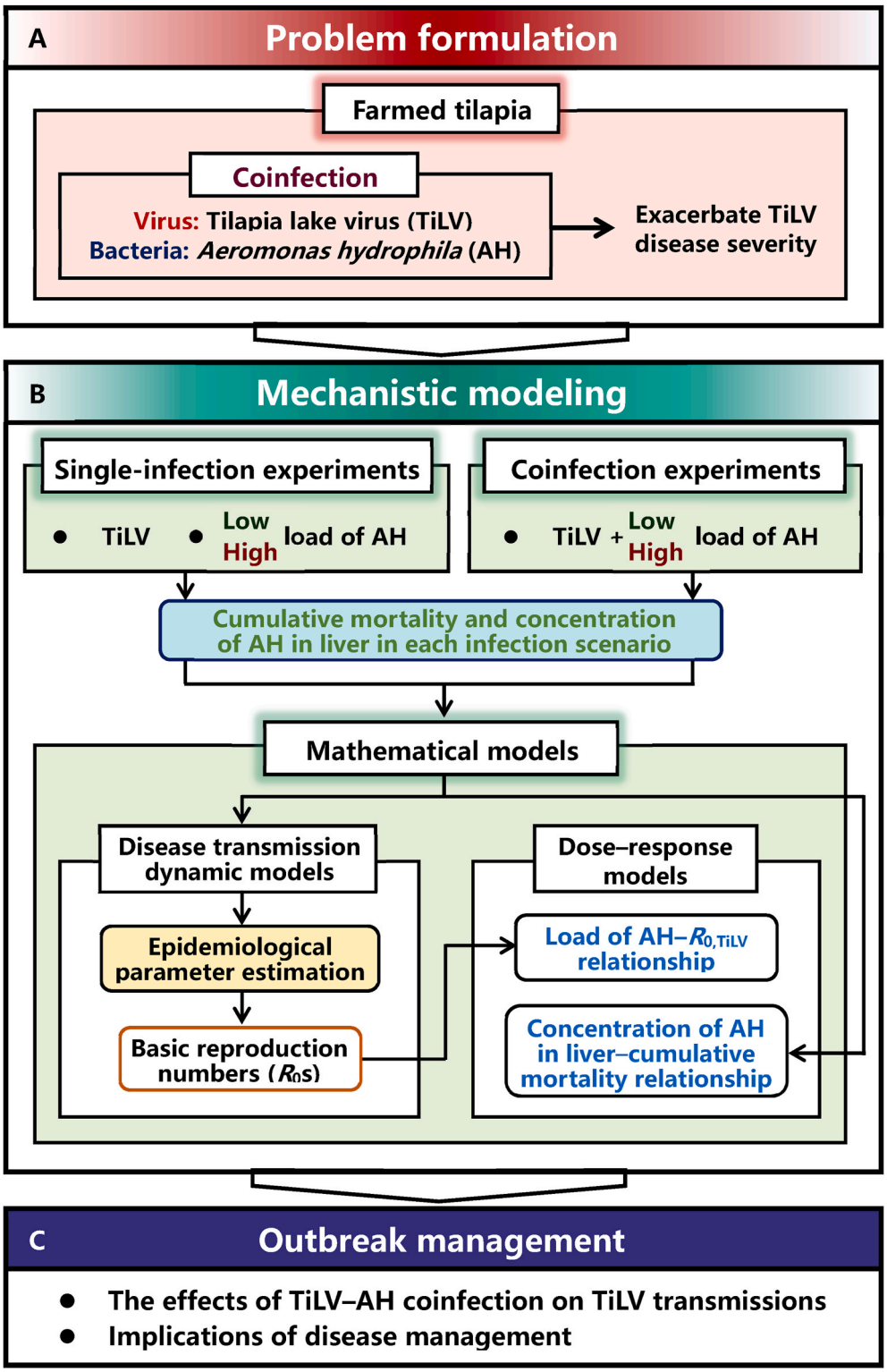

Fig. 2. Schematic systematically showing the study framework constituted by (A) problem of exacerbating TiLV disease severity formulated by coinfection, (B) mechanistic modeling process, and (C) outbreak management for AH–TiLV coinfection.

respectively (Table S3). The transmission rates due to TiLV-, AH-, and TiLV–AH-infected populations were 2.68, 2.0×10^{-3}, and 5.36×10^{-3} day^{-1}, respectively, whereas mortality rates were 0.25, 0.83, and 0.99 day^{-1}, respectively.

3.3. *Effects of TiLV–AH post-coinfection on epidemiological parameters*

The population dynamics of all states of tilapia could be simulated based on the single-infection and coinfection models and the estimated epidemiological parameters (Fig. 5). Coinfection of TiLV and high load of AH led to slightly and dramatically shorten the time, respectively, for initial TiLV- and AH-infected population appearance (Fig. 6A), whereas

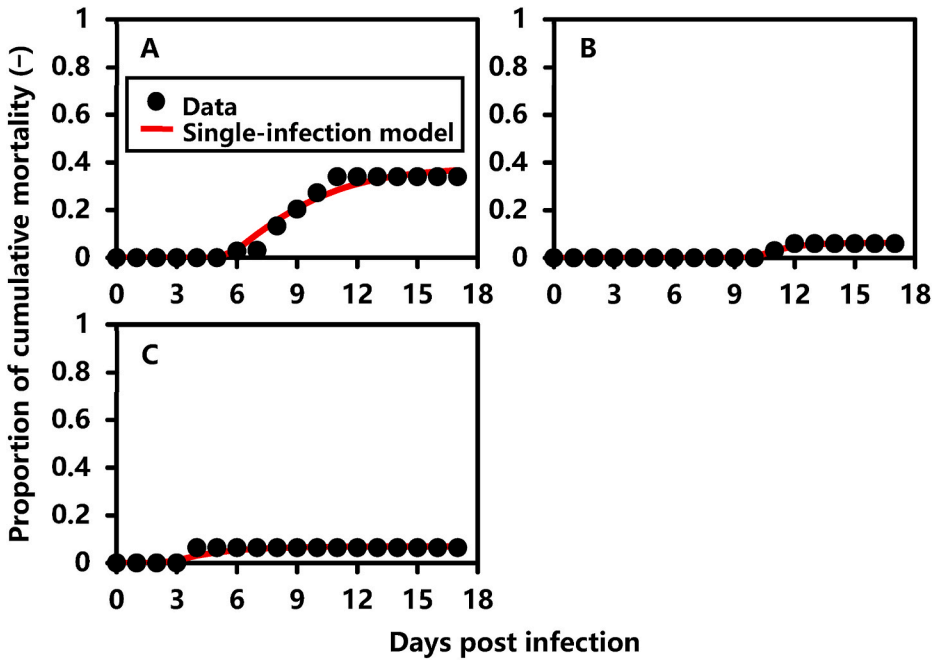

Fig. 3. Fitting the single-infection model to cumulative mortalities of tilapia infected with (A) tilapia lake virus (TiLV) (group V), (B) low load of AH (10^6 CFU fish^{-1}) (group LB), and (C) high load of AH (10^7 CFU fish^{-1}) (group HB).

generation rates of infected population were similar with values < 0.1 day^{-1} (Fig. 6B).

Although coinfection of TiLV and high load of AH could result in an increase of transmission rates among TiLV, AH, and TiLV–AH infections, rates for TiLV transmission were much higher than those in AH and TiLV–AH infections, indicating that coinfection would mainly affect the spread of TiLV rather than AH (Fig. 6C). The mortality rate for TiLV-infected tilapia was the most rapid in TiLV single-infection scenario, whereas it decreased when tilapia exposed to AH (Fig. 6D). On the contrary, mortality rate of AH-infected tilapia increased while tilapia coinfection with TiLV and AH (Fig. 6D). For mortality rate of TiLV–AH-infected population in V + HB scenario, it was two times higher than that in V + LB scenario (Fig. 6D).

Results showed that R_0 estimates for TiLV infection were all >1, whereas those for AH and TiLV–AH infections were << 1 (Fig. 6E). The estimates of R_0 for TiLV infection in groups V, V + LB, and V + HB were 1.1, 1.1, and 10.7, respectively (Fig. 6E), indicating that high load of AH imposed a dramatic increment of R_0 in TiLV transmission. Therefore, AH might play a crucial role in facilitating the spread of TiLV disease.

3.4. Bacterial load–response relationships

In groups V + LB and V + HB, after exposed to TiLV and AH, tilapia might remain susceptible or be firstly infected with AH or TiLV and then be coinfected with the other pathogen (Fig. 7A). Our results showed that for all infection states, the higher load of AH was used, the higher value of R_0 was observed (Fig. 7B,C,D). However, the trends were not the same in view of cumulative mortality dataset. While increasing load of AH, the proportion of AH infection-induced cumulative mortality decreased from 0.1 in V + LB to 0.04 in V + HB (Fig. 7B). The proportion of TiLV infection-induced cumulative mortality in V + HB was 0.86 which was extremely higher than 0.36 in V and 0.33 in V + LB (Fig. 7C). The AH–TiLV infection-induced cumulative mortality in V + HB (0.07) was seven times higher than that in V + LB (0.01) (Fig. 7D).

Since it was found that high load of AH imposed a dramatic increment of R_0 in TiLV transmission, we constructed the relationship between exposure load of AH (L_B) and R_0 for TiLV transmission ($R_{0,TiLV}$) based on R_0 estimates in groups V, V + LB, and V + HB. The response of $R_{0,TiLV}$ to increasing load of AH could be well explained by a nonlinear model: $R_{0,TiLV} = [(0.95 \pm 0.12)\ (\text{mean} \pm \text{se})]\exp[(-L_B/(-4.12 \times 10^6 \pm 2.15 \times 10^5)]$ ($r^2 = 0.99$) (Fig. 8A; Table S4).

On the other hand, a log-logistic model could well describe the relationship between concentration of AH in livers at day 5 ($C_{BL,day5}$) and cumulative mortality at day 5 (CM_{day5}) as $CM_{day5} = \frac{1}{\{1+\exp[(10.17\pm1.94)-(0.81\pm0.16)\ln C_{BL,day5}]\}}$ ($r^2 = 0.98$) (Fig. 8B; Table S4). However, when considering the infectious period of AH infection, the $C_{BL,day5}$–increased mortality (CM_{INC}) relationship was fair explained by the same log-logistic model with fitted coefficients of $c = 4.67 \pm 0.88$ and $d = 0.26 \pm 0.08$, respectively ($r^2 = 0.69$) (Fig. 8C; Table S4).

4. Discussion

4.1. Interactions of pathogens and host

Based on the constructed relationship between bacterial load and R_0 estimates, we found that AH might play a crucial role in facilitating the spread of TiLV disease; however, the interactions of TiLV and AH in host has not be elucidated and is needed to be further explored. Generally, an infection could be affected significantly by a pathogen in terms of entry and spread, rate of multiplication, ability to damage tissue, ease of transmission to other hosts, existence of animal reservoir, and drug therapy (Playfair and Bancroft, 2013). During coinfections, pathogens can compete with each other for resources or target sites inside the same host (Kotob et al., 2016). This complex interactions between pathogens and host could be synergistic or antagonistic by alterations of host susceptibility to infection, host–pathogen dynamics, infection biology, disease severity, duration of infection, and host pathology (Kotob et al., 2016).

Since TiLV and AH are different heterologous pathogens, they may be endowed with entirely different features that can influence infection and immunity. To date, tilapia was found to be the main host of TiLV (OIE, 2018; Surachetpong et al., 2020). Absence of viral receptors or mechanisms that allow virus to replicate might be the reason for most warm water fish keeping insusceptible to TiLV (Surachetpong et al., 2020). Recent studies indicated that TiLV replicates and transcripts at

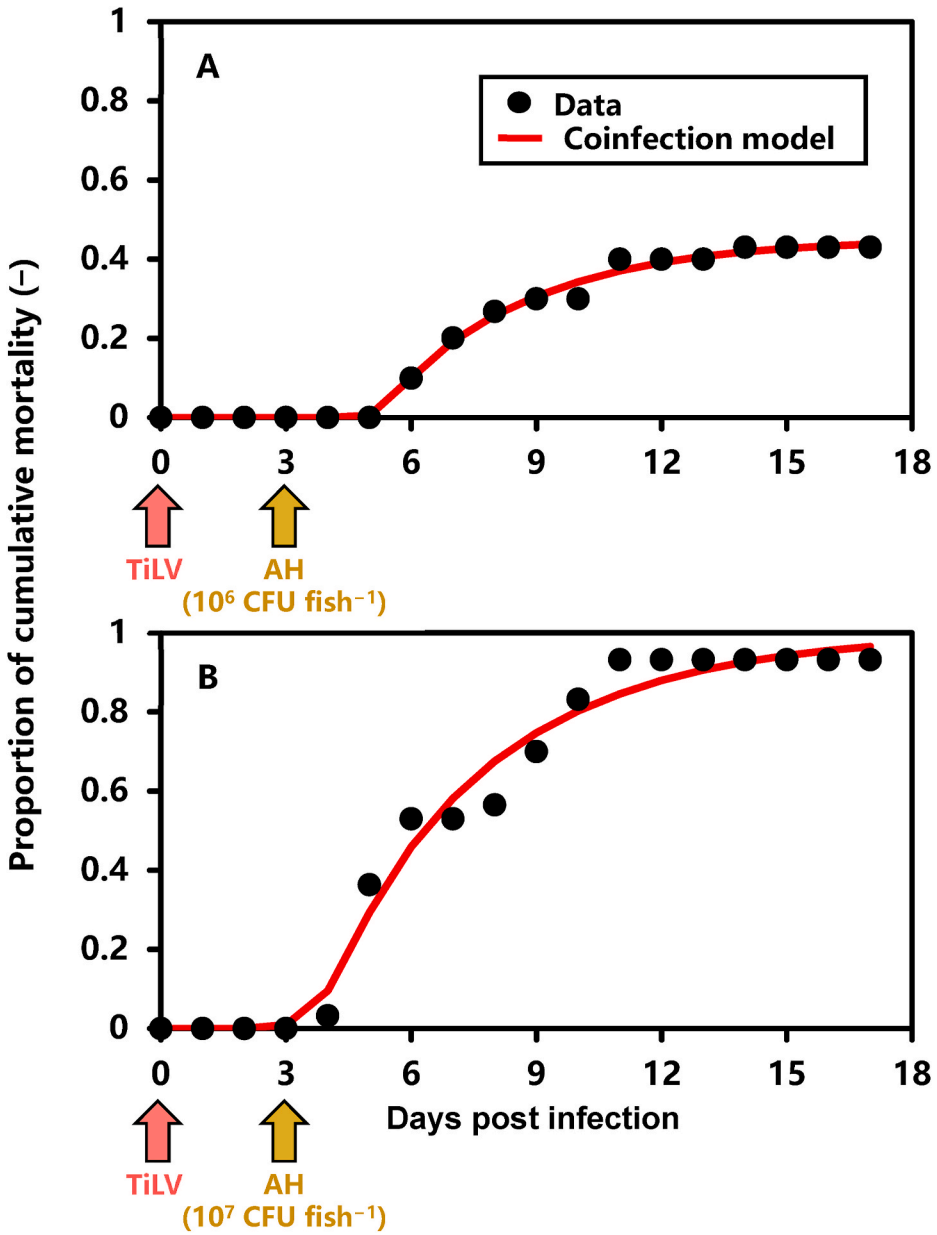

Fig. 4. Fitting the coinfection model to cumulative mortalities of tilapia infected with TiLV followed by AH at load of (A) 10^6 (group V + LB) and (B) 10^7 CFU fish^{-1} (group V + HB). (Fig. 4B), respectively. The epidemiological parameters estimated in coinfection scenario could be classified into those for the transmission due to tilapia in TiLV-, AH-, and TiLV–AH-infected states (i.e. I_V, I_B, and I_{BV} states).

the sites of pathology occurred in liver and central nervous system (Dinesh et al., 2017). The mechanisms that TiLV used for binding to and entering into host cells are suggested to be different from orthomyxoviruses despite of their morphological similarity (Chengula et al., 2019). As a virus which is not a cell and only grows within other cells by using the infected cells organelles, TiLV might be transmitted by shedding into the aquatic environment through biological fluids of fish, whereas the shedding would stop when fish is dead (Bricknell, 2017).

Differently, in addition to tilapia, a diverse host range, covering human, fish, and other aquatic animals are also affected by AH (Fernández-Bravo and Figueras, 2020; Rasmussen-Ivey et al., 2016). AH can enter fish through epithelium of intestinal tract and is recognized as an opportunistic pathogen that is capable of causing diseases in weakened fish populations or as a secondary invader in fish suffering from other diseases (CABI, 2019). Virulence factors enable AH to adhere, invade, and destroy host cell to overcome the host immune response (Fernández-Bravo and Figueras, 2020). It was found that AH could lead to massive hemocyte aggregations in hepatopancreas and fewer aggregations in gills and digestive system, indicating that hepatopancreas might be the target organ (AlYahya et al., 2018). High amount number of bacteria can be shed from fish during the acute phase of disease, and then adhere to another fish or exist in the environment for a long-term period (Bricknell, 2017). Even though host is died, bacteria can continue to grow and replicate by utilizing dead tissues as a nutrient source (Bricknell, 2017).

Additionally, a pathogen can also influence immunity covering susceptibility and escape/damage abilities to immune systems and suitability for vaccination (Playfair and Bancroft, 2013). De Chavez and Encinares (2017) indicated that AH-infected tilapia showed significantly higher white blood cell count compared to health group, indicating that AH could weaken the immunity of tilapia. Different coinfection modes of the order and the timing of infection would lead to an antagonistic or a synergistic effect due to the complex interactions between pathogens (Liu et al., 2020). While a viral infection occurs followed by a bacterial

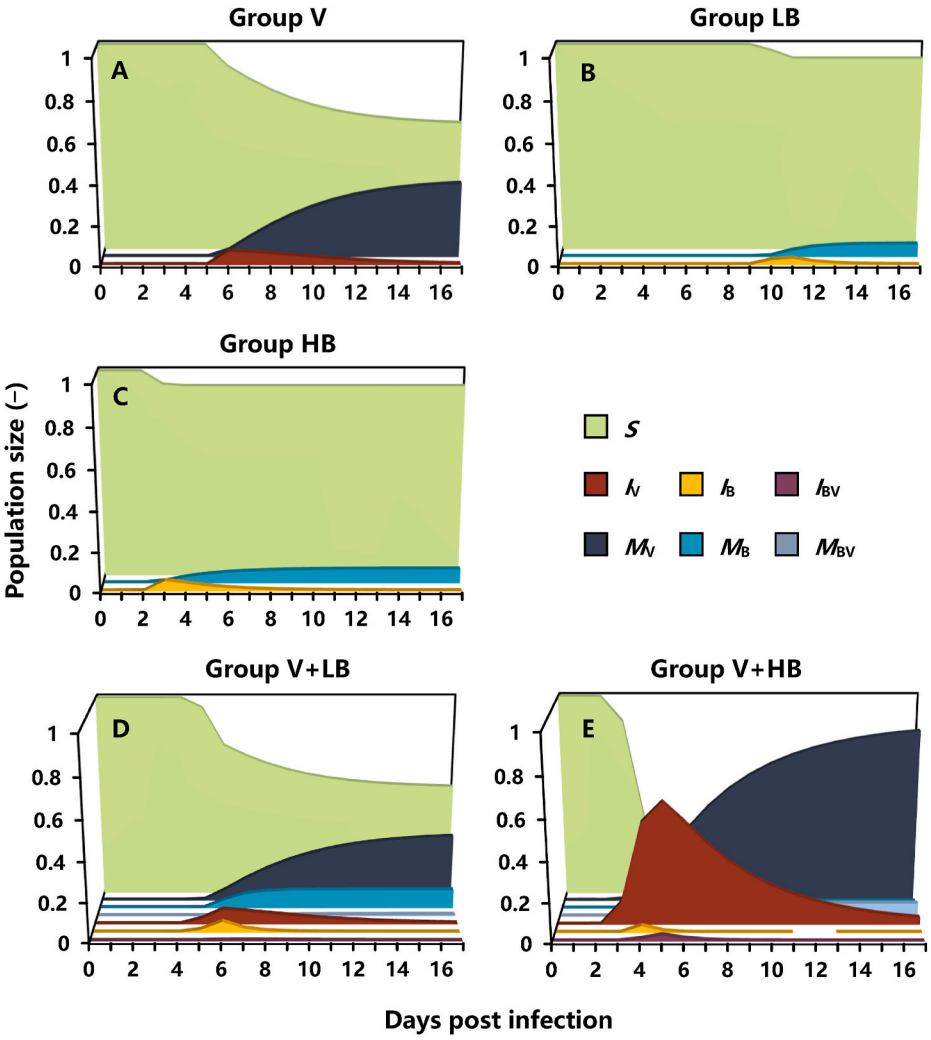

Fig. 5. Simulation of the population size of tilapia at the states of susceptible (S), TiLV-infected (I_V), AH-infected (I_B), TiLV–AH infected (I_{BV}), and mortality resulted from TiLV- (M_V), AH- (M_B), and TiLV–AH (M_{BV}) infections in groups (A) V, (B) LB, (C) HB, (D) V + LB, and (E) V + HB.

infection, the viral infection may affect the anti-bacterial immune response, increasing susceptibility of host to bacteria (Liu et al., 2020).

4.2. Effects of co-infection on TiLV transmission

In the present study, we quantified the disease epidemic in tilapia population by estimating key epidemiological parameters that played important roles in the virus−bacteria interactions dynamics. Our results showed that the initial TiLV-infected tilapia appeared 5 days post infection in population exposed to 10^4 TCID50 fish^{-1}. A recent study showed that the clinical signs had displayed in IP-injected red hybrid tilapia with TiLV concentration of 10^5 TCID50 mL^{-1} and those cohabitated with IP-injected fish within 4–5 and 9–12 days post of challenge, respectively (Tattiyapong et al., 2020). It should be notice that the higher the viral concentration is, the faster the symptoms appear. Since constrained by the available experimental data, we were unable to quantify the course of diseases for tilapia infected with TiLV at different concentrations. However, there was noteworthy evidence that R_0 estimate ($R_0 = 1.1$) in group V was lower compared with that estimated for Nile tilapia infected with TiLV at higher dose ($R_0 = 2.60 \pm 0.16$) (Yang et al., 2018).

We found that post-coinfection of AH have noticeable effects on TiLV transmission. Higher concentration of AH would shorten the time for initial AH- or TiLV-infected population appearance either in single- or co-infection scenario. Instead of increasing mortality rate, the presence of AH decreased mortality rate of TiLV-infected tilapia, whereas increased transmission rate and R_0. While the infectious period was prolonged, TiLV-infected tilapia would continuously shed the viruses, resulting in an extremely high transmissibility of TiLV disease. Although high concentration of AH would lead to high mortality for AH–TiLV-infected tilapia, it only had slight impact on the spread of diseases. It implicated that coinfection of AH and TiLV was the case of cooperative multiple diseases where the presence on one disease resulted in the other was more likely to spread (Chen et al., 2013).

Notably, our results were only for tilapia firstly infected with TiLV, and then infected with AH. Liu et al. (2020) investigated the effects of concurrent infection of AH and infectious spleen and kidney necrosis virus (ISKNV) on host and pathogens by setting the different infection models. They found that mortalities caused by secondary bacterial infection were obviously higher than those caused by secondary viral infection. It could be inferred that the transmission dynamics in the coexistence of AH and TiLV might be different due to the order of infection and was needed to be further investigated.

4.3. Implications for risk assessment and disease management

In this study, we constructed the dose–response relationships between exposure load of AH and R_0 for TiLV transmission. Although the relationships were obtained based on load of AH used for infecting tilapia via IP injection, it could be further applied−if well-designed and

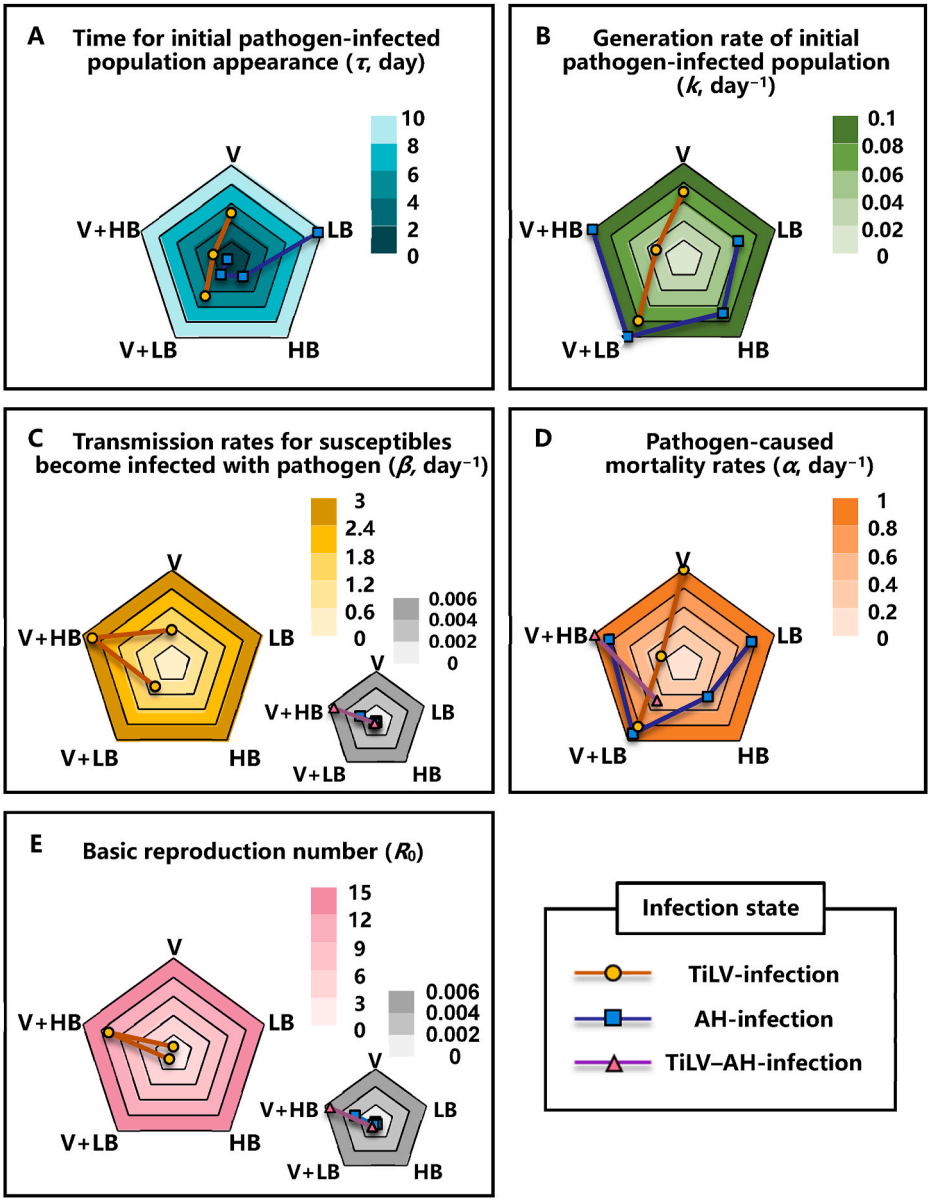

Fig. 6. Radar charts show estimates of epidemiological parameters: (A) τ, (B) k, (C) β, (D) α, and (E) R_0 represented on a color scale among TiLV-, AH-, and TiLV–AH-infections in five exposure scenarios (Single-infection groups: virus (V), low load of AH (LB), and high load of AH (HB); Co-infection groups: TiLV + low load of AH (V + LB) and TiLV + high load of AH (V + HB)). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

implemented—to assess the risk of aggravating TiLV transmission due to coexistence of AH. Especially, to date, therapy for treating TiLV disease has not been commercially available, highlighting the necessity of pursuing biosecurity measures and good management practices synergistically that might mitigate the impact of TiLV on tilapia production (Surachetpong et al., 2020). To this end, the epidemiological surveillance of the prevalence and spread of TiLV need to be comprehensively investigated (Surachetpong et al., 2020).

In light of the global threat caused by TiLV and the common presence of AH in tilapia ponds (Surachetpong et al., 2020), the impact of AH on worsening the severity of TiLV infection could not be neglectable in particular for countries that have been threaten by TiLV as well as AH. Nicholson et al. (2020) collected tilapia from 52 field outbreaks in Thailand, showing that 19% of Nile tilapia and 25% of red tilapia were concurrently infected with TiLV and *Aeromonas* spp. It was demonstrated that AH was one of the main aetiological agents affecting tilapia cultured in river-based cages in Northern Thailand (Chitmanat et al., 2016).

In Egypt, AH has been identified as the main causative agent responsible for summer mortalities in tilapia farms (Aboyadak et al., 2015; Elsheshtawy et al., 2019). AH also threatens the tilapia production in Philippines. Pakingking et al. (2015) demonstrated that AH was one of the dominant bacteria in water, sediment, and gills and intestine of tilapia cultured in brackish water earthen ponds in Philippines. They further indicated that AH constituted 33, 26, 35, and 31% of the total heterotrophic aerobic bacteria, respectively, in the water (range of counts: 10^3–10^4 CFU mL^{-1}), sediment (10^3–10^5 CFU g^{-1}), and gills (10^5–10^7 CFU g^{-1}) and intestines (10^4–10^7 CFU g^{-1}) of tilapia.

For Saudi Arabia that was labelled as with respective high risk of TiLV spread through translocation of tilapia (Dong et al., 2017a), there were studies demonstrating empirical evidence of AH. Al-Harbi and Uddin (2004) seasonally quantified bacteria in the intestine of hybrid tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) cultured in earthen ponds in Saudi Arabia. They indicated that AH was the dominant organisms with 21, 27, 15, and 17% of mean bacterial load in the ranges of 6.8×10^6–7.5×10^7, 1.6×10^6–5.1×10^7, 3.1×10^8–1.3×10^9, and 8.9×10^5–1.3×10^7 CFU g^{-1} in the seasons at mean temperatures of 28, 33, 24, and 15 °C, respectively.

They also further quantified bacterial load of the gills and intestine in brackish water tilapia (*Oreochromis niloticus*) and their surroundings

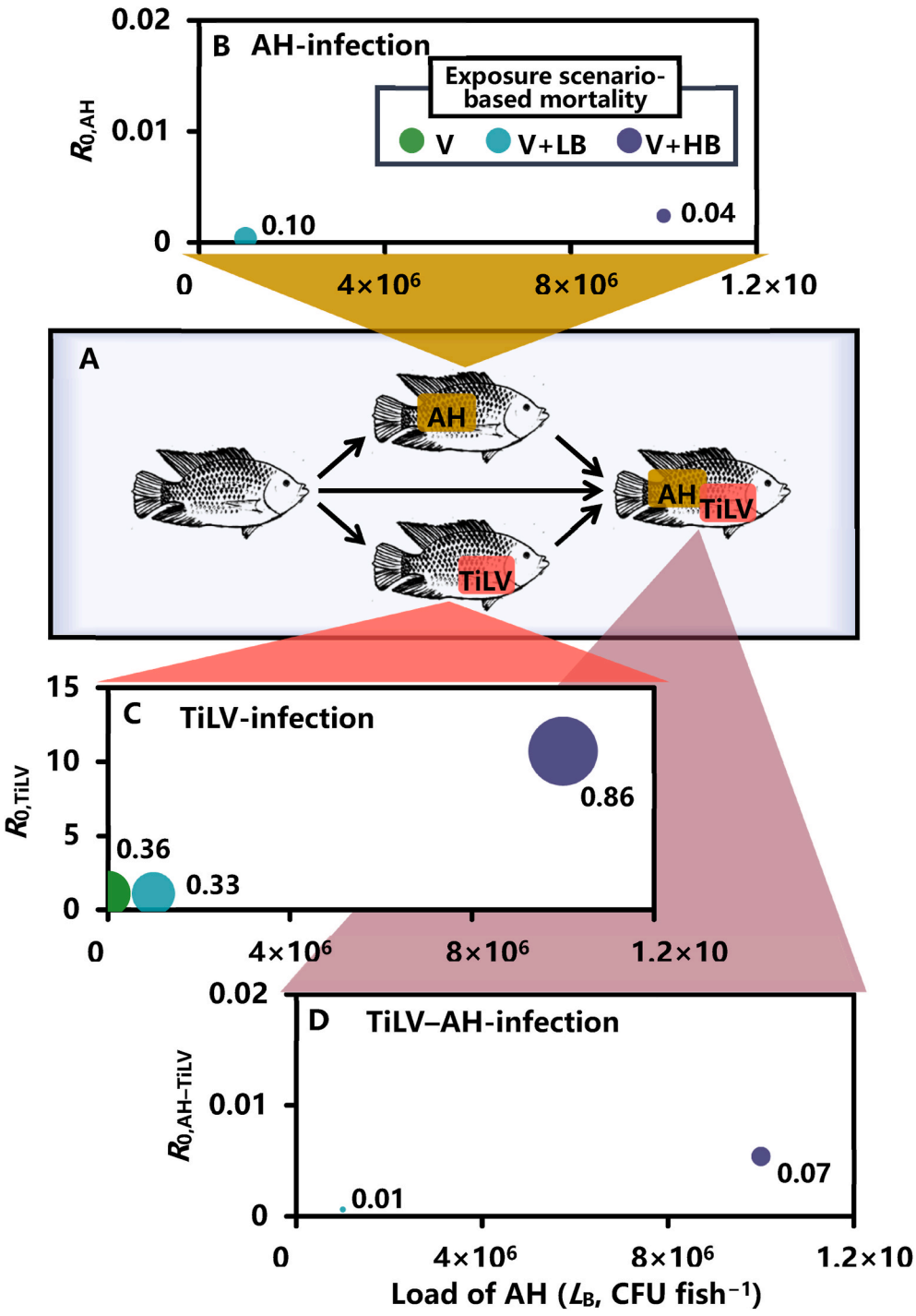

Fig. 7. (A) After exposed to TiLV and AH, tilapia might remain susceptible or be firstly infected with AH or TiLV and then co-infected with the other pathogen. The bubble chart shows the relationships among the load of AH, R_0, and cumulative mortality for (B) AH-, (C) TiLV-, and (D) TiLV–AH-infected tilapia in population along with the exposure scenarios of virus (V), TiLV + low load of AH (V + LB), and TiLV + high load of AH (V + HB). $R_{0,AH}$, $R_{0,TiLV}$, and $R_{0,AH-TiLV}$ are the basic reproduction numbers of AH-, TiLV-, and TiLV–AH-infections. The bubble size denotes the proportion of cumulative mortality.

such as pond water and sediment (Al-Harbi and Uddin, 2005). They showed that AH only accounted for 5 and 3% of total isolates in water and gills with mean bacterial loads ranging from 1.4×10^3–8.6×10^3 CFU mL^{-1} and 8.7×10^5–2.1×10^6 CFU g^{-1}, respectively. In Brazil, one of the top 12 tilapia producing countries, the prevalence of AH could range from 3 to 47% for Nile tilapia (*Oreochromis niloticus*) in cage fish farms (Rodrigues et al., 2019; USDA, 2019b).

To reduce the risk of aggravating TiLV transmission due to coexistence of AH, concentration of AH should be appropriately monitored and measures aiming at decreasing concentration of AH should also be well-implemented. Typically, antibiotics are added to feed to treat AH infection; however, exceeding use of antibiotics is highly likely to cause the potential spreading of antimicrobial resistance (AMR) (Hayatgheib et al., 2020). Therefore, alternative strategies such as vaccination, immune stimulation, phage therapy, and biosecurity approaches have been developed intensively to improve health of fish and aquaculture ecosystems and to reduce the potential spreading of AMR simultaneously (Hayatgheib et al., 2020). Incorporating the molecular techniques would be help for identifying AH-infected fish and reducing economic losses of diseases (Rodrigues et al., 2019). Moreover, since the most significant risk factor identified for the occurrence of AH was unsafe source of water with the relative risk of ~10 (Reyes, 2018), to ensuring and maintaining the water quality in farm pond is the most important actions for farmers to control concentration of AH.

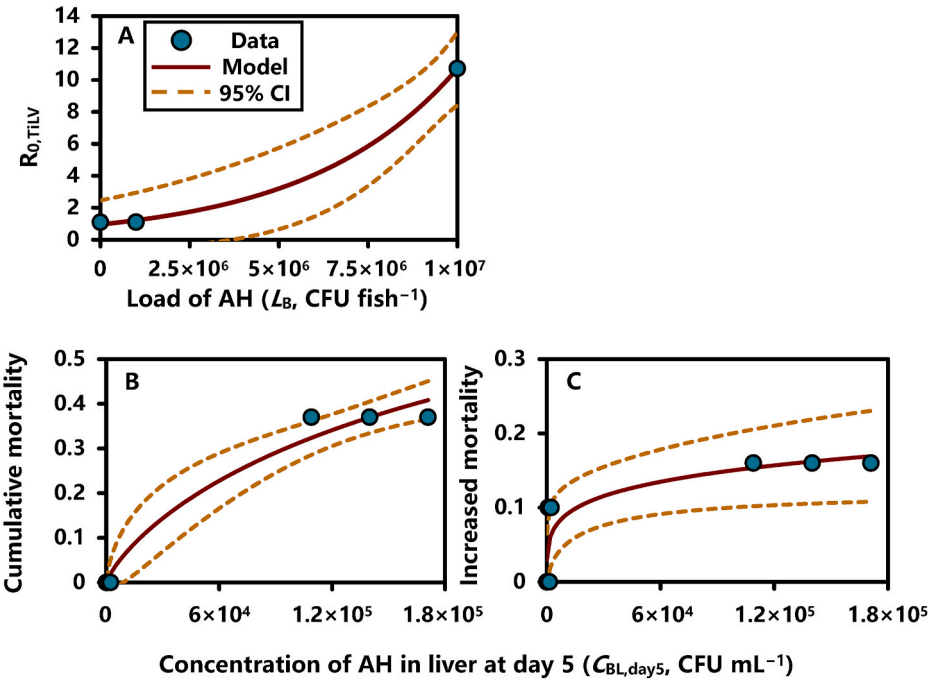

Fig. 8. (A) The relationships between the exposure load of AH (L_B) and the R_0 for TiLV transmission ($R_{0,TiLV}$) were constructed by a nonlinear model. The log-logistic model describes the relationships between (B) the concentration of AH in the livers at day 5 ($C_{BL,day5}$) and the cumulative mortality at the same day (CM_{day5}) and (C) the concentration of AH in liver at day 5 ($C_{BL,day5}$) and the increased cumulative mortality (CM_{INC}) observed between the same day and the day after an infectious period (i.e., the inverse of α_B).

5. Conclusions

Owing to the characteristics of wide distribution in aquatic environment, AH concentration in fish pond can be a key environmental indicator for assessing exacerbating mortality risk of TiLV-infected tilapia under confection. Our findings provide useful insights for research in three major ways. First, we quantified the effect of bacterial and viral coinfection with different heterologous pathogens of TiLV and AH on the population dynamics of tilapia. This could form a mechanistic basis towards depicting the impact of virus−bacteria interactions in the coinfection dynamics on tilapia aquaculture. Second, our findings highlight that load of AH and R_0 for TiLV transmission are the effective indicators for ensuring the substantial dose−response relationships that could be employed in further risk assessment task. Third, our findings could inform farm managers to underscore the necessity of taking positive actions to reduce the risk of aggravating TiLV transmission during the globally emerging threat caused by TiLV in the coming decades. Overall, our analysis establishes and quantifies the link between the TiLV-induced mortality and the coinfection dynamics in fish, with implications for virus−bacteria interactions-driven disease management and control.

Data availability statement

Datasets of proportion of cumulative mortality for tilapia after single infection with TiLV, low load of AH, or high load of AH, and coinfection with TiLV + low load of AH or TiLV + high load of AH were extracted from Nicholson et al. (2020).

Nicholson, P., Mon-on, N., Jaemwimol, P., Tattiyapong, P., & Surachetpong, W. (2020).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.indic.2021.100135.

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