

Comparison of Growth Parameters of Pathogenic and Nonpathogenic *Vibrio parahaemolyticus* on Cooked Shrimp

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The present research is to compare the growth parameters of six pathogenic and non pathogenic *Vibrio parahaemolyticus* (*V. parahaemolyticus*) strains on shrimp at 37 °C. The *V. parahaemolyticus* counts on inoculated cooked shrimp (*Litopenaeus vannamei*) was enumerated. The growth parameters of pathogenic *V. parahaemolyticus* (pVp) and nonpathogenic *V. parahaemolyticus* (npVp) were obtained by fitting growth data to the modified Gompertz model. The specific maximum growth rate (μ_{max}) of *tdh* gene positive (*tdh*⁺) pVp Vp2 (*tdh*⁺/*trh*), Vp6 (*tdh*⁺/*trh*), Vp16 (*tdh*⁺/*trh*) was 2.17, 2.32 and 1.7 (log₁₀ CFU/g)·h⁻¹ respectively. The μ_{max} of *trh* gene positive (*trh*⁺) pVp Vp3 (*tdh*/*trh*⁺) and Vp 19 (*tdh*/*trh*⁺) was 1.24 and 1.8 (log₁₀ CFU/g)·h⁻¹ respectively, The μ_{max} of npVp Vp18 (*tdh*/*trh*) was 1.95 (log₁₀ CFU/g)·h⁻¹. The lag time (\bar{e}) of *tdh*⁺ pVp Vp2, Vp6 and Vp16 was 0.55, 1.04 and 0.83 h respectively. The \bar{e} of *trh*⁺ pVp Vp3 and Vp19 was 0.13 and 0.39 h respectively, the lag time (\bar{e}) of npVp Vp18 was 0.32 h. The maximum population density (N_{max}) of *tdh*⁺ pVp Vp2, Vp6 and Vp16 was 9.03, 9.43 and 9.12 log₁₀ CFU/g respectively. The N_{max} of *trh*⁺ pVp Vp3 and Vp19 was 9.19 and 9.22 log₁₀ CFU/g respectively, The μ_{max} of npVp Vp18 was 9.42 log₁₀ CFU/g. It is revealed that the clinical *tdh*⁺ pVp grow faster than the npVp, *trh*⁺ pVp and food isolated *tdh*⁺ pVp. The study could contribute to the strain selection in predictive model construction and provide important information for government to set effective management measures to ensure food safety.

Key words: Comparison, growth parameters, pathogenic and nonpathogenic *Vibrio parahaemolyticus*, shrimp.

Vibrio parahaemolyticus is recognized as the leading cause of seafood-borne illnesses in China (Liu *et al.* 2004). Shrimp is frequently contaminated by *V. parahaemolyticus* (DePaola *et al.* 2003; FAO/WHO 2011; Chen *et al.* 2012), consumption of cross-contaminated or

undercooked seafood contaminated by *V. parahaemolyticus* can increase the risk of human illness (Su *et al.*, 2007). However, cooked shrimp also cause food poisoning cases (FAO/WHO 2011; Sani *et al.* 2012). The risk assessment of *V. parahaemolyticus* is ¹ about several kinds of seafood, like oysters, bloody clam, finfish and cooked shrimp (Yamamoto *et al.* 2008; Iwahori *et al.* 2010; FAO/WHO 2011; Sani *et al.* 2012).

The exposure assessment is the main component of risk assessment, and one of the main issues in exposure assessment is the predictive

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model. The strain selection is crucial to the model development. However, there is little information about the growth difference between pathogenic and nonpathogenic *V. parahaemolyticus* strain, some study pointed out that the nonpathogenic *V. parahaemolyticus* grow faster than the pathogenic *V. parahaemolyticus* (Yoon *et al.* 2008), on the contrary, the other found that growth rate of pathogenic *V. parahaemolyticus* was greater than total *V. parahaemolyticus* (Mudoh *et al.* 2010), as the prevalence of pathogenic strain in food sample is always very low (Zhao *et al.* 2011), which could mean that the growth rate of pathogenic *V. parahaemolyticus* was greater than nonpathogenic *V. parahaemolyticus*. The risk assessment of *V. parahaemolyticus* of seafood are based on the assumption that the growth rate of pathogenic and non-pathogenic strains is the same (FAO/WHO 2011).

Considering the limited information and the different findings of previous study, it is necessary to study the viability of different

nonpathogenic and pathogenic *V. parahaemolyticus*, which contains the thermostable direct hemolysin (*tdh*⁺) or the thermostable direct hemolysin-related hemolysin (*trh*⁺) genes. Therefore, the present study was to compare the growth parameters of six different pathogenic and nonpathogenic on shrimp.

MATERIALS AND METHODS

Strains and inoculum preparation

Six pathogenic and nonpathogenic *V. parahaemolyticus* strains are preserved at our laboratory at -80°C in tryptone soy broth (Beijing Land Bridge Technology Company Ltd., Beijing, P.R.China) containing 3% NaCl (pH 7.4) with addition of 25% (vol/vol) sterilized glycerol as a cryoprotector. For each experiment, each *V. parahaemolyticus* strain was transferred from -80°C storage and was sub-cultured to stationary phase. Details of six *V. parahaemolyticus* are shown in Table 1.

Table 1. Six *V. parahaemolyticus* in the present study

Name	<i>tth</i>	<i>tdh</i>	<i>trh</i>	Isolation
Vp2	+	+	-	ATCC, Gastroenteritis, Maryland
Vp3	+	-	+	ATCC, Shirasu food poisoning, Japan
Vp6	+	+	-	Clinical isolation, Gastroenteritis patient isolation, China
Vp16	+	+	-	Isolated from fresh <i>Macrobrachium nipponense</i> in Shanghai market
Vp18	+	-	-	Isolated from fresh <i>L.vannamei</i> in Shanghai market
Vp19	+	-	+	Isolated from fresh <i>L.vannamei</i> in Shanghai market

Preparation and inoculation of the cooked shrimp

Live shrimp (*L.vannamei*) was purchased from a local aquaculture pond in Pudong New District, Shanghai, China in October 2011. The live shrimp was transported to our laboratory at Shanghai Ocean University in plastic bags containing pond water sparged with oxygen to keep the shrimps viable. At the laboratory the shrimps were rinsed with running tap water, a protocol described in GB/T 4789.7-2008, National Standards China. The rinsed whole shrimps were then packed into sterile sampling bags and stored at -80°C , two bags of which were thawed overnight at 4°C before use.

The shrimp were placed into boiling water for 30 min and transferred to a biosafety hood immediately to cool the shrimp under room temperature aseptically. The whole shrimp (about 13 ± 2 g per sample) was selected and dipped in *V. parahaemolyticus* suspension of $5 \log_{10}$ CFU/ml for 20 min with shaking. The shrimp were air-dried in a biosafety hood for 20 min to allow for bacterial attachment. This treatment ensured the *V. parahaemolyticus* on the whole shrimp was approximately $4 \log_{10}$ CFU/g.

Growth on cooked shrimp at 37°C and bacterial enumeration

The inoculated shrimp were placed into

sterile sealed plastic bags (Beijing Land Bridge Technology Company Ltd., Beijing, P.R.China) and stored under isothermal conditions in high precision low temperature incubators (model MIR 154; Sanyo Electric Co.,) at 37 °C. Samples was taken out each hour and were placed in a sterile 400–ml filter stomacher bag (Beijing Land Bridge Technology Company Ltd., Beijing, P.R.China) with 100 ml of sterile alkaline peptone water (APW, Beijing Land Bridge Technology Company Ltd., Beijing, P.R.China) with 3 % NaCl (pH 8.0), and then the sample was stomached (BagMixer400, Interscience, France) for 2 min. 0.1ml of 10-fold serial dilutions of shrimp homogenates was spread onto thiosulfate-citrate-bile salts-sucrose (TCBS) in Petri dishes for enumeration of *V. parahaemolyticus* and incubated at 37 °C for 18 h - 24 h. Three replicates of at least two appropriate dilutions depending on the sampling time were enumerated. For each test, the absence of *V. parahaemolyticus* on non-inoculated shrimp was also confirmed by TCBS plate count and none of tested un-inoculated samples was found of *V. parahaemolyticus*.

Modeling growth parameters of six different stains of Vp on cooked shrimp

The means of *V. parahaemolyticus* counts (log₁₀ CFU/g) were graphed as a function of time. The growth curves for each strain were fitting to the modified Gompertz model (Mataragas et al. 2006)

$$N_t = A + C \times \exp\{-\exp[-B \times (t - M)]\} \dots(1)$$

Where: N_t is the cell concentration at time t (h) (log₁₀ CFU/g), A is the initial cell concentration (log₁₀ CFU/g) C is the difference between the upper asymptotic line of the growth curve (maximum population density, N_{max}) minus the lower asymptotic line. B is the relative maximum growth rate at time M, M is the time at which the growth rate is maximum (h). Then, μ_{max} (log₁₀ CFU/g)·h⁻¹ and λ (h) can be calculation by the following

equations:

$$\mu_{max} = \frac{BC}{e} \dots (2)$$

$$\lambda = M - \frac{1}{B} \dots(3)$$

$$N_{max} = A + C \dots(4)$$

The growth curve fitting was performed using the software ORIGIN 7.5.

Statistical analysis

The performance of modified Gompertz equation to the growth data was evaluated by the coefficient of determination (R²).

RESULTS AND DISCUSSION

The growth of six different *V. parahaemolyticus* was shown in Fig 1. The coefficient of determination of the data to the modified Gompertz model, for the six *V. parahaemolyticus* strains 2, 3, 6, 16, 18, 19 was 0.97, 0.99, 0.99, 0.98, 0.99, 0.99 respectively, which indicated that the modified Gompertz model is adequately good to be applied to estimate the growth parameters of these *V. parahaemolyticus* strains.

As shown in Table 2, the mean lag time of these six stains on cooked shrimp at 37 °C was 0.543±0.339 h, which is similar to the lag time of *V. parahaemolyticus* under fresh prawn (0.698 h) (Boonyawantang et al. 2012). The mean maximum population density of these six *V. parahaemolyticus* strains is 9.235±0.161 log₁₀ CFU/g, which is higher than the maximum population density (8.28 log₁₀ CFU/g) of *V. parahaemolyticus* on salmon (Yang et al. 2009) and the maximum population density (6 log₁₀ CFU/g) of *V. parahaemolyticus* in oysters (FAO/WHO 2011). It is could be due to the pH difference between shrimp, oyster and salmon, the pH of oyster is

Table 2. Growth parameters of 6 different *V. parahaemolyticus* strains

Growth Parameters	Vp2	Vp3	Vp6	Vp16	Vp18	Vp19
μ _{max}	2.17	1.24	2.32	1.7	1.95	1.8
λ	0.55	0.13	1.04	0.83	0.32	0.39
N _{max}	9.03	9.19	9.43	9.12	9.42	9.22

between 4.8-6.3, the pH of fish is between 6.6-6.8, but the pH of shrimp in the present study is around 8, which is closer to the optimum pH (7.6-8.6) of *V. parahaemolyticus* (Jay et al. 2005).

The μ_{max} of Vp2, 3, 6, 16, 18, 19 was 2.17, 1.24, 2.32, 1.7, 1.95 and 1.8 respectively. The μ_{max} of the fastest strain in the present study, Vp6 which possesses *tdh* gene grew nearly 2 times faster than the Vp3 which possesses *trh* gene. It is evident that the growth rate of these pathogenic and nonpathogenic *V. parahaemolyticus* was different from each other. The nonpathogenic *V. parahaemolyticus* in the present study is Vp16 (*tdh*/*trh*⁻), which was isolated fresh *L.vannamei* in Shanghai market. The μ_{max} of Vp16 is higher than Vp3(*tdh*/*trh*⁺), Vp19(*tdh*/*trh*⁺) and Vp16(*tdh*⁺/*trh*⁻), but lower than Vp2(*tdh*⁺/*trh*⁺) and Vp6(*tdh*⁺/*trh*⁺). It could be concluded that μ_{max} of the nonpathogenic *V. parahaemolyticus* is greater than the *trh* gene positive *V. parahaemolyticus* on shrimp, the similar result was reported by (Yoon et al. 2008), while, this research just compare the growth between pathogenic *V. parahaemolyticus* (*tdh*/*trh*⁺) and nonpathogenic *V. parahaemolyticus*, which didn't consider pathogenic *V. parahaemolyticus* (*tdh*⁺/*trh*⁻). Another interesting finding in the present study is nonpathogenic (*tdh*/*trh*⁻) stains grew slower than the clinical isolated pathogenic strain(*tdh*⁺/*trh*⁻), but faster than the food isolated pathogenic (*tdh*⁺/*trh*⁺), which could be indicated that the clinical pathogenic *V. parahaemolyticus* could grow faster

than the nonpathogenic and food pathogenic *V. parahaemolyticus*, which emphasized the importance of strain selection for the purpose of *V. parahaemolyticus* predictive model development. There isn't exact information about the difference between the growth rate of pathogenic and nonpathogenic *V. parahaemolyticus*, therefore, the risk assessment of *V. parahaemolyticus* involved in some assumption that total and pathogenic *V. parahaemolyticus* grow at the same speed (FAO/WHO 2011) or pathogenic and nonpathogenic strains present the same growth rate (Yamamoto et al. 2008), which is considered to be one of the data gaps in the present risk assessment of *V. parahaemolyticus*.

The difference of different pathogenic and nonpathogenic strain is a source for intra-species variability of bacterial growth parameters, which may influence the accuracy of risk assessment (Sant'Ana et al. 2012). Therefore, it is necessary to provide more information about growth parameters of different pathogenic and nonpathogenic *V. parahaemolyticus* stains to minimize the uncertainty of risk assessment results. This research was conducted under 37°C, which is the optimum temperature of *V. parahaemolyticus* (Miles et al. 1997). At 37°C, the six *Vibrio parahaemolyticus* showed variation between growth parameters of different strains, the growth variation between *V. parahaemolyticus* strains under other growth temperatures and more strains growth parameters should be studied in the future.

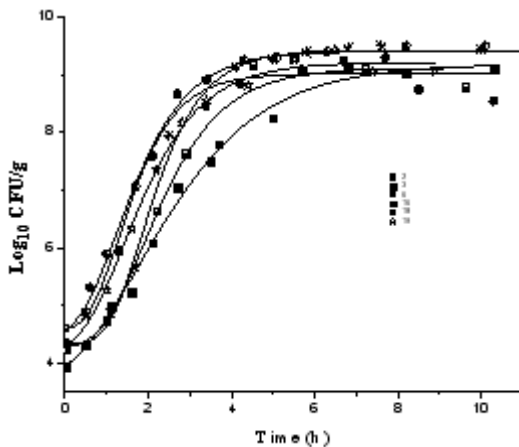


Fig. 1. Growth curves of strains of different *V. parahaemolyticus* at 37 °C

CONCLUSION

The pathogenic and nonpathogenic *V. parahaemolyticus* appears to show different growth parameters. The finding in the present study could provide important information to the strain selection in predictive model construction to keep fail-safe prediction of *V. parahaemolyticus* growth. On the other hand, it is also very important to be noticed in quantitative risk assessment of *V. parahaemolyticus*, the risk of illness could need to be determined using growth rates of pathogenic strains instead of the total population or nonpathogenic strains and the time between cooked and preservation should need to be shortened.

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