

Expansion of the time-to-turbidity model for proteolytic *Clostridium botulinum* to include spore numbers

A model was previously developed to predict the time-to-turbidity of proteolytic Clostridium botulinum spores placed in broths having various pH values, NaCl concentrations and temperatures (Whiting and Call 1993); however, the model had a constant number of spores (10^4). This fixed number of spores limits the use of the model and makes inferences to foods more difficult. New data were collected and fitted to the logistic model. Regression equations were calculated to provide parameter estimates for different combinations of temperature, pH, NaCl concentration and spore number. The predicted mean times-to-turbidity increased with decreasing numbers of spores; at 20°C-pH7.0-1.0% NaCl, for example, the mean time-to-turbidity was 4.3 and 7.1 days for 10^4 and 10^0 spores per sample, respectively.

Introduction

Observance of the times for the appearance of turbidity, haze or gas in a sealed tube containing spores of *Clostridium botulinum* permits the collection of sufficient data to model the influence of multiple variables on germination and growth. A logistic (sigmoidal) model for the fraction of samples exhibiting growth with time was developed for proteolytic strains of *C. botulinum* (Whiting and Call 1993). The environmental factors in the model were pH, NaCl concentra-

tion and temperature. A subsequent model for nonproteolytic B strains showed the major effect that the number of spores in the sample had on the observed times (Whiting and Oriente 1997). As the number of spores decreased, the mean time-to-turbidity increased markedly, but the variation also increased. Because information on the effect of spore numbers is important to a user of these models, a new data set was collected for the proteolytic strains and new equations were calculated with spore numbers as an additional factor.

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Materials and Methods

The procedure was similar to that described previously (Whiting and Call 1993). A six-

strain mixture of three type A (69, FDA; 62, FDA; 33, US Army Lab., Natick, Massachusetts) and three proteolytic type B (169, FDA; 999, FDA; ATCC 7949) spores was prepared. Spores of each strain were individually grown in 500 ml Reinforced Clostridial Medium (RCM, Difco) at 35°C inside an anaerobic chamber (Coy Laboratory Products, Inc., Ann Arbor, Michigan) for 3 weeks. The spore cultures were centrifuged at 5860 *g* for 15 min, washed with sterile water and resuspended in 50% ethanol. The spores from each strain were enumerated by being plated on Brain-Heart Infusion agar (Difco) and incubated at 35°C inside the anaerobic chamber. Equal numbers of spores from each strain were then mixed to form a spore mixture containing 3×10^6 spores ml⁻¹ and stored at 4°C.

Batches of RCM were adjusted by adding NaCl (0.5–4.5% w/v) and/or 0.1N HCl (pH 5.0–7.2), and 10 ml were dispensed into culture tubes. Immediately after being autoclaved, the tubes were placed inside the anaerobic chamber to cool overnight. An aliquot of the spore mixture was heat shocked (10 min at 80°C), dilutions were made with sterile water and the tubes with 10 ml RCM were each inoculated with 0.1 ml of the diluted spores (10^0 , $10^{1.5}$, $10^{2.5}$ or 10^4 spores per tube). The inoculated tubes were capped with sterile VASPAR, removed from the anaerobic chamber and immediately placed in incubators at 15, 19, 26 or 34°C. The tubes were observed for signs of visible growth twice daily for 3 days, daily up to 3 weeks and three times per week for as long as 60 days. The procedure was repeated four times in a fractional factorial design and the observations were combined. A total of 111 combinations of temperature, pH, salt and spore numbers were observed. Each combination had 10 (favorable conditions) or 20 (less favorable conditions) tubes. The total number of tubes was 1230, plus occasional uninoculated tubes to confirm sterile handling and to serve as a visual standard.

An estimate of the probability of growth at a given time was calculated as the number of tubes showing evidence of growth in each treatment combination, divided by the total

number of tubes in that treatment. The probability estimate as a function of time was fitted to a logistic function

$$P_t = P_{max} / (1 + e^{k(\tau - t)}) \quad (1)$$

where P_t is the probability (0 to 1) at time t , P_{max} , is the maximum probability of growth after 60 days, k is the rate constant (day⁻¹) and τ is the time of the midpoint of the function (days) (Whiting and Call 1993). The observations were fitted to the logistic function using RS/1 (BBN Software Products Corp., Cambridge, Massachusetts). The effect of the four treatments (temperature, pH, NaCl concentration and spore numbers) on the three parameters (P_{max} , k and τ) was modeled by polynomial regression analysis (SAS Institute, Inc., Cary, North Carolina). To calculate the regression equation for τ , the treatment combinations with no growth were removed and the log₁₀ τ was used. The 95% confidence intervals for τ were also calculated (Draper and Smith 1998).

Results and Discussion

Parameter values (P_{max} , k and τ) for selected treatment combinations are presented in Table 1 (the complete data set is available upon request). Where no turbidity or other indication of growth was observed for the entire 60 days, P_{max} , and k are 0.0 and τ is blank. The fitting of P_{max} was not bounded and the best fit was allowed to give values slightly greater than 1.0 for some treatments. The rate term, k , was limited to 10.0, when the set of tubes went from no growth to all tubes being turbid in less than 2 days. Data showed that increasing the spore numbers generally increased P_{max} , and decreased τ . They also showed that increased temperatures or pH values or decreased salt levels decreased the τ times.

The regression equations (Table 2) were all significant ($P < 0.01$). However, their ability to explain the variations was not always substantial. The k value had a low r^2 ; therefore, the simplified equation obtained by backwards regression was used ($r^2 = 0.27$).

Table 1. Fitted P_{max} , k and τ values for 14 of the 111 treatment combinations and the P_{max} , τ and lower confidence limit (LCL) for τ calculated from the regression equations

pH	NaCl (%)	Temp (°C)	Spores (log)	Fitted parameter values from individual sets of tubes			Calculated parameter values from regression equations		
				P_{max}	k (day ⁻¹)	tau (days)	P_{max}	tau (days)	LCL tau (days)
7.2	0.5	15	0.0	0.72	0.7	14.0	0.87	19.1	12.3
7.2	0.5	15	1.5	1.04	2.0	10.9	0.95	14.1	9.8
7.2	0.5	15	4.0	1.00	7.2	9.0	0.86	13.4	8.4
7.2	0.5	19	4.0	0.90	9.8	6.6	0.97	4.5	3.0
7.2	2.5	19	4.0	0.77	6.4	11.9	0.66	9.3	6.4
6.5	1.5	19	1.5	1.00	8.3	10.6	0.55	8.6	6.9
6.5	1.5	26	1.5	0.88	2.1	2.4	0.64	2.7	2.1
6.5	1.5	34	1.5	1.00	4.5	1.0	0.68	1.5	1.1
5.5	1.5	26	0.0	0.10	10.0	8.0	0.17	9.4	6.8
5.5	1.5	26	1.5	0.40	9.2	3.9	0.44	6.3	5.0
5.5	1.5	26	2.5	1.00	8.1	4.8	0.56	5.4	4.2
5.5	1.5	26	4.0	1.00	1.1	4.0	0.66	5.0	3.9
5.5	1.5	19	0.0	0.00	0.0		0.14	23.4	7.1
5.5	1.5	19	4.0	0.15	2.7	31.9	0.36	15.7	11.9

The relatively large variation about this parameter was also observed in the earlier version of this model (Whiting and Call 1993) and for the nonproteolytic *C. botulinum* model (Whiting and Oriente 1997). However, the impact of this parameter on this model was not great and it did not have practical importance. The P_{max} parameter indicated the probability that a tube will eventually (within 60 days) show growth; it is an estimate of the probability that at least one spore in the tube will germinate and grow. In most instances, at treatment combinations

where no growth was observed ($P_{max} = 0.0$), the regression equation estimated a low value for P_{max} . For some treatment combinations, the equation calculated a value less than zero; this is also interpreted as a combination with a low probability of growth.

The τ parameter is the most important parameter of the model and estimates the time for the median number of the positive tubes to show turbidity. The observed values ranged from 1 to 53 days. For most treatment combinations, the tubes that would become turbid did so within a relatively short time

Table 2. Regression equations for P_{max} , k and τ

$$P_{max} = 0.6980 + 0.04364 T - 0.4958 \text{pH} - 0.1509 \text{NaCl} + 0.01310 \text{Sp} - 0.005863 T \times \text{pH} \\ + 0.01121 T \times \text{NaCl} + 0.009903 T \times \text{Sp} - 0.02631 \text{pH} \times \text{NaCl} - 0.01069 \text{pH} \times \text{Sp} \\ - 0.000524 \text{NaCl} \times \text{Sp} - 0.000541 T^2 + 0.07580 \text{pH}^2 - 0.007928 \text{NaCl}^2 - 0.02189 \text{Sp}^2$$

$$r^2 = 0.67$$

$$n = 111$$

$$k = 3.0464 - 0.5396 \text{NaCl} - 2.6720 \text{Sp} + 0.07898 T \times \text{pH} + 0.5615 \text{pH} \times \text{Sp} - 0.2207 \\ \text{NaCl} \times \text{Sp} - 0.008476 T^2 - 0.1660 \text{pH}^2$$

$$r^2 = 0.27$$

$$F = 5.4$$

$$n = 111$$

$$\text{Log}_{10} \tau = 7.7406 - 0.1202 T - 1.2392 \text{pH} - 0.1586 \text{NaCl} - 0.04214 \text{Sp} - 0.01038 T \times \text{pH} \\ - 0.000284 T \times \text{NaCl} - 0.003427 T \times \text{Sp} + 0.03264 \text{pH} \times \text{NaCl} - 0.003637 \text{pH} \times \text{Sp} \\ + 0.003721 \text{NaCl} \times \text{Sp} + 0.002689 T^2 + 0.09148 \text{pH}^2 + 0.02325 \text{NaCl}^2 + 0.01969 \text{Sp}^2$$

$$r^2 = 0.89$$

$$n = 79$$

T, temperature (°C); NaCl, percentage NaCl; Sp, log number of spores.

lated non-zero values for τ at treatment combinations where no turbidity was observed. These treatment combinations usually had relatively wide confidence intervals and also low estimates for P_{max} . The value calculated from the regression equation for P_{max} with 10^2 spores was 0.57 which decreased with 10^0 spores to 0.15 (30°C, pH 5.0 and 3.5% NaCl). This provides a quantitative indication of how both the likelihood of growth as well as the time for growth vary with different combinations of environmental conditions and spore numbers.

The inoculation of exactly 10^0 or 1 spore per tube cannot be achieved, instead a Poisson distribution of spores results. This means that 63% of the tubes would contain one or more spores and 37% of the tubes would have none. The data for 1 spore per tube with favorable growth conditions indicated growth in about 70% of the samples. This distribution would not affect the τ times but would be a component of the regression equation for P_{max} .

In summary, this model provides an estimate of the time for germination and growth

of proteolytic *C. botulinum* for specified temperatures, pH values, added NaCl and number of spores. Interpretation of the P_{max} and confidence intervals for τ provides an indication of the uncertainty in this estimation. This model is incorporated into the USDA's Pathogen Modeling Program which may be obtained by contacting the corresponding author.

References

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