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Prolonged Incubation Period of Salmonellosis Associated with Low Bacterial Doses

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ABSTRACT

In gastroenteritis outbreaks caused by *Salmonella*-contaminated lunches at elementary, junior high, and nursery schools, outbreaks with long median incubation periods (i.e., 60 to 120 h) were observed frequently between 1990 and 1999 in Japan. We analyzed epidemiological data on 185 outbreaks of *Salmonella* Enteritidis infection to study the factors underlying the long incubation period. These survey results showed that the median incubation period for *Salmonella* Enteritidis infection from contaminated school and nursery school lunches was significantly longer than that from other types of cooking facilities. In addition, we analyzed the relationship between the median incubation period and the bacterial dose ingested per person in nine outbreaks of *Salmonella* Enteritidis infection; the bacterial dose was estimated with reference to the bacterial concentration in the causative foods. A significant negative correlation between the bacterial dose ingested per person and the median incubation period is clearly shown. The time elapsed from the start of the cooking process to the consumption of school and nursery school lunches was significantly shorter than at other cooking facilities, suggesting limited bacterial growth, which in turn is thought to lead to a long incubation period.

The number of outbreaks and patients suffering from foodborne *Salmonella* infection increased from 1990 to 1999 in Japan. The number of outbreaks has tended to decrease thereafter; however, *Salmonella* is even now the main causative pathogen of foodborne disease. Therefore, taking preventive measures against foodborne *Salmonella* infection is an important food hygiene issue in Japan, as is the case for many other countries. For investigations into the foods that caused *Salmonella* infection and the facilities involved, the incubation period is critical because it usually determines the data and time length of food consumption surveys in interviews with the patients.

The incubation period for *Salmonella* infection is generally reported to be 8 to 24 h, ranging from 6 to 48 h *(4)*. However, much longer incubation periods (i.e., 60 to 120 h) are frequently observed in outbreaks of gastroenteritis caused by *Salmonella* Enteritidis (SE)–contaminated school lunches of elementary and junior high schools (hereafter referred to as school lunches) and nursery schools in Japan. Oda et al. *(9)* reported that the median incubation period for foodborne infection caused by SE-contaminated nursery school lunches was as long as 108 h, and the number of ingested cells per patient was estimated to be small, 23 to 39 cells per person. Matsui et al. *(6)* reported that the median incubation period for foodborne infection caused by SE-contaminated dessert buns in Toyohasi City in 2001 was 168.0 h, and the number of cells ingested per patient was estimated to be below the detection limit by the most-probable-number (MPN) method (i.e., less than 30 cells per 100 g). Moreover, several other studies indicated that the incubation periods for some *Salmonella* infections were longer than is generally accepted *(1, 2, 8, 10, 12)*. The relationship between the prolonged incubation period and factors affecting this prolongation, including the number of cells ingested and the method of cooking, has not been analyzed thoroughly.

According to the Food Sanitation Law of Japan, local health centers of prefectures and government-designated major cities report to the prefectural governors the results of epidemiological investigations in the form of the ''Food Poisoning Investigation Report.'' In these reports, the results of detailed investigations into items such as incubation periods, symptoms in patients, cooking methods of the causative foods, causative pathogens, and contamination routes are indicated. We collected these reports and used them to analyze the factors leading to a prolonged incubation period.

MATERIALS AND METHODS

Foodborne SE infection outbreaks used as study subjects. Among the investigation reports collected from 39 prefectures and 9 government-designated major cities in Japan from 1982 to 2002, 185 outbreaks caused by SE had data for incubation periods and microbiological tests. These outbreaks fulfilled the following requirements: (i) the number of patients in the outbreak was 10 or more, (ii) fecal cultures were positive for SE and negative for other pathogens, and (iii) causative meals or dishes were identified on the basis of microbiological tests or through interviews with the patients regarding foods eaten before the onset of illness.

Investigated items at different cooking facilities. Following the classification made by the Japanese Ministry of Health,

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Labor, and Welfare, we grouped the causative facilities as follows: elementary and junior high schools, nursery schools, restaurants, take-out food shops, hotels, and hospital and welfare facilities. We investigated patient gender and average age, median incubation period, attack rate, and the time elapsed from the start of the cooking process to consumption of the SE-contaminated food (hereafter referred to as elapsed time) for each facility group. No leftover or reserved foods had been served among the cases examined, but in one case, stored foods were served the day after. In this case, the elapsed time was calculated from the start of the preparation period.

From the nine outbreaks that provided data on SE concentrations in causative foods, the following data sets are summarized in Table 1: vehicle foods, cooking method, number of patients, attack rate, median incubation period, SE concentration in vehicle foods, amount of food intake per person, SE dose ingested per person, and food conditions before bacterial examination. The same method used in past studies could not be adopted here because testing was conducted by different municipalities. However, we were able to select nine cases for which bacterial tests were conducted at the central institute of each municipality with reliable test accuracy. In collecting these data, we selected cases with reliable data in which the preservation temperatures of foods were recorded and unclear preservation temperatures were excluded and cases in which foods had been preserved at room temperature. The laboratory techniques used for bacterial isolation and enumeration are also described in the footnotes of Table 1.

Calculation of median incubation period. In the investigation reports, each patient is tabulated in terms of incubation period (every 6 to 24 h). We selected the middle time of each incubation period range as the representative value, and the median incubation period was calculated from the representative value of the range.

Estimation of SE dose ingested per person. The SE dose ingested per person was estimated with the use of the SE concentration in the vehicle food multiplied by the amount of food intake per person. Although determining the food intake per person is difficult because of individual differences, we used the food intake per person data as determined by the schools or nursery schools that had prepared the menus firsthand. For other institutions, numerical values calculated by public health centers were used. Because pupils of schools and nursing schools are strongly encouraged to clear their plates, we calculated the intake per person by assuming that they cleared their plates. If the ingested doses were reported in the investigation reports, those values were used.

Determination of elapsed time. Elapsed time was defined as the period between the start of the cooking process, including cooking procedures such as cutting, boiling, or steaming, and the time of consumption of the cooked food. Because vegetables mixed with seafood and dressed with vinegar or other seasonings, such as salad (i.e., dishes that are not heated whole), were frequently the causative foods in school lunches, the time period from the beginning of the food preparation process to serving was surveyed, deeming it essential to take the increase in bacteria during food preparation into consideration. This information was also obtained from the investigation reports.

Statistical analysis. The relationship between the median incubation period and the ingested dose per person and between the median incubation period and the attack rate were analyzed by regression analysis. Comparison of the median incubation period, the attack rate, and elapsed time between groups was by one-way analysis of variance. The level of significance of the degree of risk was set at less than 5% ($P < 0.05$).

RESULTS

Number and gender of patients for all cooking facilities. The total number of patients whose data were used in the investigation was 27,463. Sex was identified for 27,376 (99.7%) patients, of which 14,833 (54.2%) were male and 12,543 (45.8%) female. There was no relationship between the male:female ratio and the median incubation period for different cooking facilities.

Average age of patients for different cooking facilities. Average age of patients classified according to cooking facility was 55.3 years for food prepared in hospital and welfare facilities; 40 years for food prepared in restaurants, take-out food shops, and hotels; 10.6 years for school lunches; and 4.5 years for nursery school lunches.

Median incubation period for different cooking facilities. Median incubation periods for foodborne SE infection in each kind of cooking facility are shown in Table 2. The median incubation period of the various groups ranged from 24.0 to 28.4 h for food prepared in restaurants (C), take-out food shops (D), hotels (E), and hospital and welfare facilities (F), whereas median incubation period was 80.9 h for school lunches (A) and 64.8 h for nursery school lunches (B). When the median incubation period was compared among the groups, it was significantly longer for school and nursery school lunches than for food prepared in other cooking facilities ($P < 0.01$). Furthermore, the incubation periods between food prepared in restaurants, take-out food shops, hotels, and hospital and welfare facilities were not significantly different. Figure 1 shows the distribution of the median incubation period for SE infection outbreaks. The distribution of incubation periods was broader for school and nursery school lunches than for the other groups.

Relationship between median incubation period and ingested dose per person. Table 1 shows the ingested dose per person and the median incubation period in nine outbreaks. In outbreak 1, the SE concentration in the causative food (dessert bun) was reported at less than 30 cells per 100 g by the MPN method *(6)*. On the basis of this SE concentration in the food, the SE dose ingested per person for one dessert bun was estimated to be less than 12 cells, the weight of a dessert bun being 40 g. For the analysis, 12 cells were used. In outbreak 2, the ingested dose per infant less than 4 years old and per 5-year-old child was estimated as 23 and 39 cells, respectively *(9)*. Therefore, the average value, 31 cells, was used as the ingested dose per person for this outbreak. For other outbreaks, the ingested dose per person was calculated with the use of the SE concentration in the food and the amount of food intake per person. As shown in Figure 2, a negative correlation was found between the ingested dose per person and the median incubation period ($y = -74.049 \log x + 151.6$, $r = 0.933$, $P < 0.01$).

TABLE 1. *Relationship between the median incubation period and ingested SE dose estimated with the use of bacterial quantitative data of causative foods*

Outbreak	Vehicle	Cooking method	Causative facilities	No. of patients	Attack rate (%)	Median incubation period (h)	SE concentration (g^{-1})	Food intake (g/person)	SE dose ingested (CFU/person)	Food condition
1	Dessert buns	Chestnut paste wrapped with jelly made from tapioca and then steamed	Elementary and junior high school	171	Unknown	168.0	$<0.3^a$	40	12	Frozen for 77 days
2	Egg salad	Boiled macaroni, cucumber, carrot, ham, and canned corn, dressed with mayonnaise	Nursery school	42	26.9	108.0	0.78^b	40	31	Frozen for 9 days
3	Spinach with peanut dressing	Boiled spinach, carrot, and peanuts, dressed with sauce	Elementary and junior high school	644	12.1	96.0	1.4^c	35	49	Frozen for 7 days
4	Beef and bean sprouts with sesame dressing	Boiled beef with seasoning, boiled bean sprout, and carrots dressed with sesame oil and soy sauce	Elementary and junior high school	967	9.2	94.5	4×10^d	22	8.8×10^2	Refrigerated
5	Boiled vegetables with sauce dressing	Boiled spinach, cabbage, bean sprouts, carrots, and scrambled eggs, dressed with sauce	Elementary and junior high school	107	23.0	78.0	10^e	100	1×10^3	Frozen for 5 days
6	Three foods with sauce dressing	Boiled spinach, stewed carrots, deep-fried bean curd, and omelet, dressed with sauce	Nursery school	26	59.1	48.0	23^f	40	9.2×10^2	Frozen for 7 days
7	Scallops boiled with cream sauce	Steam steeped scallops in sake broiled with egg, oil, and salt in oven	Hotel	30	79.0	32.5	$2.0 \times 10^{4\,g}$	50	1.0×10^6	Refrigerated for 9 days
8	Cabbage, seachicken, and harusame, dressed with sauce	Cabbage, seachicken, and sticks of bean jelly, stir-fried with sauce	Elementary and junior high school	69	52.3	26.0	$6.0 \times 10^{2\,h}$	100	6.0×10^4	Refrigerated for 2 days
9	Bavarois	Mixed hot milk with egg yolk, whipped cream, sugar, and gelatin, subsequently cooled	Junior college	100	81.3	22.5	$3.0 \times 10^{3\,i}$	111	3.33×10^5	Refrigerated for 1 day

[a] Sample (10 g) diluted to 1:10, then five-tube MPN method with EEM, SBG, and DHL were used.
[b] Sample (10 g) diluted to 1:10, then five-tube MPN method with EEM, SS, and DHL used.
[c] Sample (10 g) diluted to 1:10, then five-tube MPN method with EEM, SBG, and DHL were used.
[d] Sample (0.1 ml of 10-g sample diluted 10 times) was plated on manitol lysine crystal violet brilliant green agar.
[e] Sample (10 g) diluted to 1:10, then five-tube MPN method with selenite broth and SS were used.
[f] Sample (5 g) diluted to 1:10, then three-tube MPN method using EEM, SS, and DHL.
[g] Diluted sample was directly plated.
[h] Sample (10 g) diluted to 1:10, then five-tube MPN method used.
[i] Sample (0.1 ml of 10-g sample diluted 10 times) was plated on DHL.

TABLE 2. *Median incubation period classified according to kind of causative cooking facilities*

Institution[a]	No. of incidents	Median incubation period (h)	SD	Groups showing significant associations[b]			
A	35	80.9	35.876	C	D	E	F
B	17	64.8	21.583	C	D	E	F
C	46	24.0	8.557	A	B		
D	50	26.7	10.887	A	B		
E	27	28.4	10.736	A	B		
F	10	26.6	12.186	A	B		
Total	185	—	—				

[a] A, elementary and junior high school lunches; B, nursery school lunches; and food prepared in: C, restaurants; D, take-out food shops; E, hotels; F, hospitals and welfare facilities.

[b] Differences in incubation period among six groups were examined by Scheffe's test after ANOVA ($P < 0.05$).

Attack rate for different cooking facilities. Mean attack rate by cooking facility was 46.1% for school lunches (33 incidents), 47.2% for nursery school lunches (17 incidents), 58.6% for restaurant foods (44 incidents), 51.2% for take-out foods (51 incidents), 49.6% for hotel foods (26 incidents), and 48.5% for hospital and welfare facility foods (10 incidents). However, no statistical correlation was observed between attack rates and type of facility.

Relationship between median incubation period and attack rate. As a result of the regression analysis of 50 food poisoning cases caused by school and nursery school lunches, a negative correlation was observed between the attack rate and the median incubation period ($y = -0.746x + 106.33$, $r = 0.491$, $P < 0.01$). Also as a result of the regression analysis of six cases limited to school and nursery school lunches of the nine cases shown in Table 1, a negative correlation was observed between the attack rate and the median incubation period ($y = -1.11x + 109.957$, $r = 0.856$, $P = 0.03$).

Elapsed time for different cooking facilities. In the Japanese school lunch system, foodstuffs are brought into the kitchens early in the morning of the day of use or in the afternoon of the day before and are used only for school lunches. These foodstuffs were cooked from 8:30 a.m. to noon in the kitchens and were eaten by 1 p.m. Although the nursery school lunches also were prepared by a similar process, the time for cooking in nursery schools is generally shorter than in other schools, the lunches being eaten between 10 a.m. and noon. Table 3 shows the average elapsed time for different cooking facilities. The average time between the start of the cooking process and consumption of food prepared by restaurants, take-out food shops, hotels, and hospital and welfare facilities ranged from 10.8 to 21.8 h. However, the required cooking times were short for school and nursery school lunches—4.8 and 2.9 h, respectively. The variability of data for restaurants, take-out food shops, hotels, and hospital and welfare facilities was greater than that for school and nursery school lunches. The average elapsed time of school lunches and nursery school

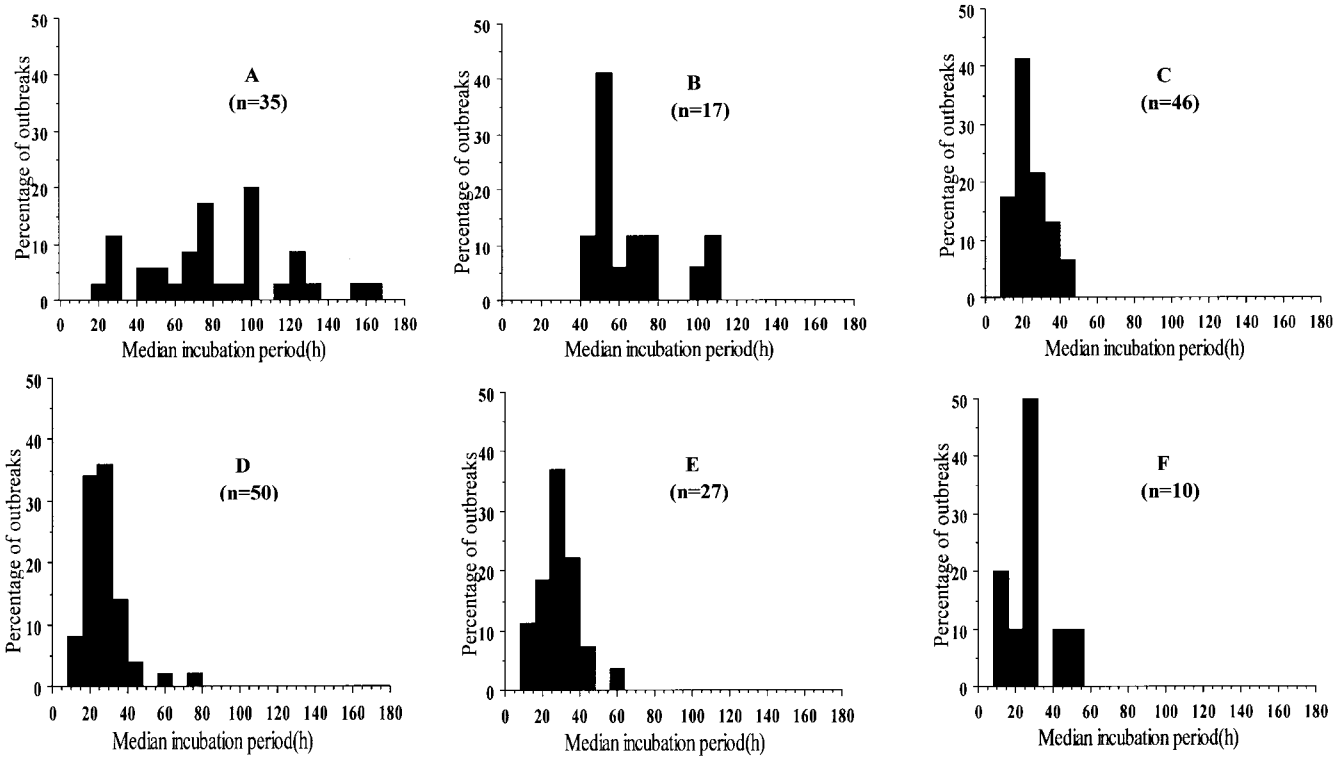

FIGURE 1. *Distribution of median incubation period of SE outbreaks (*n *= 185) classified according to kind of causative cooking facilities: (A) elementary and junior high school lunches, 35 outbreaks; (B) nursery school lunches, 17 outbreaks; food prepared in (C) restaurants, 46 outbreaks; (D) take-out food shops, 50 outbreaks; (E) hotels, 27 outbreaks; and (F) hospital and welfare facilities, 10 outbreaks.*

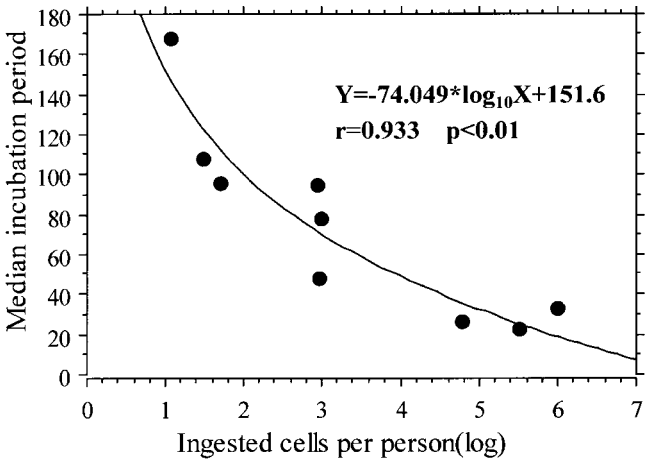

FIGURE 2. *Relationship between the median incubation period and the logarithmic bacterial dose ingested per person by nonlinear regression analysis.*

lunches was significantly shorter than that of food prepared by restaurants, take-out food shops, and hotels. However, no significant difference was observed in the average elapsed cooking time in restaurants, take-out food shops, hotels, and hospital and welfare facilities.

DISCUSSION

Gastroenteritis outbreaks caused by school and nursery school lunches have frequently been observed to have median incubation periods of more than 70 h, significantly longer than that for contaminated food prepared at other cooking facilities. Recognizing this, we analyzed the relationship between median incubation period and ingested dose per person in nine outbreaks of SE infection estimated with the use of SE concentrations in the causative foods (Table 1). According to the results of this survey, in outbreak 1 (168.0 h), with the longest median incubation period, the SE dose ingested per person was estimated at less than 12 cells. In addition, outbreaks 2 through 6 had median incubation periods longer than 48 h; the SE dose ingested per person in each outbreak was estimated at between 31 and about 10^3 cells.

Staff were advised to keep cooked foods as well as raw materials frozen at temperatures below −20°C until microbiological examination at large-scale cooking facilities could be performed under guidance provided by the Director General of the Environmental Sanitation Bureau of the Japanese Ministry of Health and Welfare. Freezing at −20°C for 90 days can leave the number of SE either unchanged or reduced by 1 log cycle *(5).* Because the implicated foods were kept frozen below −20°C for 2 to 77 days or were refrigerated for 1 to 2 days until they were subjected to microbiological examination, it is reasonable to assume that the number of SE did not change significantly between the moment of consumption and the moment of enumeration and that the SE dose ingested per person estimated with the use of SE concentrations in the causative foods is credible. In a *Salmonella* Typhimurium infection study by Glynn and Palmer *(3),* the incubation period was affected by the amount of contaminated food ingested; the mean incubation period (20.7 h) for a group that ingested a small amount of contaminated foods was significantly longer than that (16.6 h) for a group that ingested more than twice the amount of contaminated foods. In addition to this report, similar reports show that the incubation period is affected by the amount of contaminated food ingested, although these data are within the range of the incubation period for common *Salmonella* infections *(7, 11).* These results could be explained by the assumption that the incubation period of *Salmonella* infections is affected by the bacterial dose ingested. In addition, through our analysis here, we demonstrated that the median incubation period is prolonged to more than 48 h when the SE dose ingested per person is less than about 10^3 cells.

As a result of the analysis of 50 food poisoning cases caused by school and nursery school lunches, a negative correlation was observed between the attack rate and the median incubation period. Furthermore, the regression analysis of six cases limited to school and nursery school lunches for which bacterial concentrations could be determined also showed a negative correlation between attack rate and median incubation period. In short, the attack rates were low in SE food poisoning cases with long incubation periods. The above results also suggest that, in food poisoning cases caused by *Salmonella* in which bacteria doses are low, attack rates are low and incubation periods are long.

Because we observed that the long incubation period for SE infection was related to the characteristics of the cooking methods of school and nursery school lunches, we analyzed the data of each outbreak, focusing on the elapsed time between the start of the cooking process and the time of consumption. According to the results of this survey, the average elapsed time was 4.8 h for school lunches and 2.9 h for nursery school lunches. These periods were significantly shorter than those for food prepared in restaurants (19.2 h), take-out food shops (17.4 h), and hotels (21.8 h). A shorter cooking process and time period immediately be-

TABLE 3. *The time elapsed from the start of cooking to consumption of the contaminated food classified according to kind of causative cooking facilities*

Institution[a]	No. of incidents	Average elapsed time (h)[b]	SD	Groups showing significant associations[c]		
A	34	4.8	7.024	C	D	E
B	19	2.9	0.871	C	D	E
C	33	19.2	13.622	A	B	
D	44	17.4	10.621	A	B	
E	20	21.8	16.126	A	B	
F	10	10.8	2.835			
Total	160	—	—			

[a] A, elementary and junior high school lunches; B, nursery school lunches; and food prepared in: C, restaurants; D, take-out food shops; E, hotels; F, hospitals and welfare facilities.

[b] Elapsed time was calculated as the period from the start of cooking to the time of consumption of cooked food.

[c] Differences in elapsed time among six groups were examined by Scheffe's test after ANOVA ($P < 0.05$).

fore serving allows less time for the proliferation of bacteria; therefore, the number of ingested cells is probably very small.

The previous discussion leads to the assumption that the short elapsed time characteristic of school lunches and nursery school lunches affected SE outbreaks, resulting in prolonged incubation periods. Because it is possible that food ingredients or competitive bacteria have an effect on the survival and growth of *Salmonella*, it is necessary to perform more detailed investigations into the characteristics of the methods of cooking in causative facilities.

The attack rate in food poisoning cases caused by *Salmonella* is greatly affected by age of the infected person. Here, we noted that the average age of patients affected by contaminated food prepared in restaurants, take-out food shops, hotels, and hospital and welfare facilities was 37 to 55 years. On the other hand, many of the *Salmonella* infection victims at elementary and junior high schools and nursery schools were young—less than 10.6 years old. It is possible that the age of an individual also contributes to a long incubation period. We plan to study the relationship between age and incubation period further.

The attack rate between food poisoning cases in schools and nursery schools and cases in other categories of facilities were not different, despite a longer incubation period for the school and nursery school category, possibly because food poisoning remained latent at the other facilities because the attack rate is low for adults in food poisoning cases with long incubation periods. In future studies, we will proceed with an analysis of the effects of age and other factors. In this study, we gathered and analyzed only SE cases in order to eliminate, as much as possible, the effects of the difference in pathogenicity by *Salmonella* serotype on the infectious dose. However, differences in virulence by strain could be present. This possibility should be a subject of future research.

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