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Original Article

Analysis of *Bordetella pertussis* Agglutinin Titers during an Outbreak of Pertussis at a University in Japan

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SUMMARY: In 2007, a large outbreak of pertussis occurred at a university in Japan. Initially, a student, suffering from nocturnal cough and post-tussive vomiting for 3 weeks was diagnosed with pertussis. During the subsequent outbreak, 361 university students and staff members presented with a primary complaint of a cough. In the present study, we analyzed bacterial agglutinin titers against two *Bordetella pertussis* strains, Yamaguchi (epidemic strain) and Tohama (vaccine strain), in 310 patients with a cough and evaluated its diagnostic accuracy for adolescent and adult pertussis. These serological analyses showed a significant difference ($P < 0.001$) in the levels of Yamaguchi agglutinin titer, but not in those of Tohama agglutinin titer, between patient and healthy adult groups. Therefore, the bacterial agglutination assay against strain Yamaguchi may be a useful tool for diagnosis of adolescent and adult pertussis, especially in young adults, when an agglutinin titer cutoff value of ≥160× is used in combination with clinical symptoms and other clinical laboratory tests.

INTRODUCTION

In Japan, more than 100,000 cases of pertussis were reported every year before the 1950s. Whole-cell pertussis vaccine was introduced in 1950 in Japan, followed by a dramatic decrease in the number of pertussis cases (1). In the 1970s, it was reported that the whole-cell pertussis vaccine caused encephalitis in Japan, and the pertussis vaccination rate in Japan then decreased, followed by an increase in the number of pertussis cases (1). A safer purified diphtheria-tetanus-acellular pertussis (DTaP) vaccine was introduced in Japan in 1981. Consequently, the prevalence of pertussis decreased and Japan was reported to reach the WHO pertussis target of a prevalence of <1/100,000 persons (2), although this conclusion was questionable. In 2007, there was a large-scale pertussis outbreak in adolescents and adults at a university in Japan, during which we were able to analyze *Bordetella pertussis* agglutinin titers. To the best of our knowledge, this is the first report of an analysis of *B. pertussis* agglutinin titers among adolescents and adults during a pertussis outbreak.

PATIENTS AND METHODS

Serological tests: Analysis of *B. pertussis* agglutinin titers was performed on people with a cough during a pertussis outbreak at the university (outbreak group: students and staff members $n = 310$; 176 male, 134 female; age range 18–55 years; average age 23.7; median age 21). Bacterial agglutinin titers against *B. pertussis* strains Yamaguchi (epidemic strain, agglutinogens 1, 3, 6, 7, and 13) and Tohama (vaccine strain, agglutinogens 1, 2, 4, 7, and 13) were measured using the *B. pertussis* antigen for agglutination test 'SEIKEN' N (Denka Seiken, Tokyo, Japan) according to the manufacturer's instructions (3). The cut-off point for a positive result was taken as an agglutinin titer ≥40×. This criterion is used for serological diagnosis for infants and children in Japan. To determine the cut-off criterion for adult pertussis, we compared data from the 2007 university outbreak group with data from healthy people without a cough during the same season (4) (control group $n = 246$; 156 male, 90 female; age range 21–60 years; average age 40.3; median age 39.5).

Genetic tests: Molecular diagnosis of *B. pertussis* infections was performed using the loop-mediated isothermal amplification (LAMP) method (5). Nasopharyngeal swab specimens were collected from 60 patients with suspected pertussis, and total DNA was extracted using QIAamp DNA Micro Kits (Qiagen, Hilden, Germany). The DNA samples (2 μl) were analyzed by the LAMP assay.

Case definition: A "probable case" was defined as a person who had a cough and a *B. pertussis* strain Yamaguchi agglutinin titer ≥40× between May 17 and July 4, 2007. A "definite case" was defined as a person who had a cough, a *B. pertussis* strain Yamaguchi agglutinin titer ≥40×, and a positive LAMP assay result between May 17 and July 4, 2007.

Statistical analyses: The Mann-Whitney U-test, chi-square test, and t test were performed. The level of significance was $P < 0.01$.

RESULTS

Progression of the pertussis outbreak: On May 17, 2007, a student complained about a prolonged severe cough and was diagnosed with pertussis based on the clinical findings at a university hospital. On the following day, another stu-

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dent complained about a severe cough that had persisted for 4 weeks and was diagnosed with pertussis based on the clinical findings at a general hospital. On the same day, 10 students complained of a persistent cough and, on May 24, 8 of the 10 students were diagnosed with pertussis. Also on May 24, the mass media reported a pertussis outbreak at the university. Starting the next day, many students and staff members began to complain of a persistent cough, and the number of pertussis patients continued to increase. It was agreed that an outbreak was spreading throughout the entire university, and it was decided to close the university for 2 weeks. Patients diagnosed with pertussis were treated with macrolides. The last patient presented on July 4 (Fig. 1). From May 17 to July 4, 361 people (285 students, 76 staff members) had a primary complaint of a cough, and 290 of these (231 students, 59 staff members) were serologically-positive: 270 were diagnosed as "probable cases" and 20 as "definite cases." Chemoprophylaxis with macrolides was provided to 1,163 persons who had contact with pertussis patients and who would come in contact with infants due to hospital or educational practices (6,7). We analyzed the symptoms and life-styles of students and staff members with severe cough and found that these patients' contacts clustered around three specific places: a dormitory, a club, and an office.

Cases in the dormitory: In the dormitory, a student complained of a nocturnal cough, paroxysmal cough, dyspnea, and post-tussive vomiting for 3 weeks. Five students in the dormitory subsequently complained of a paroxysmal cough

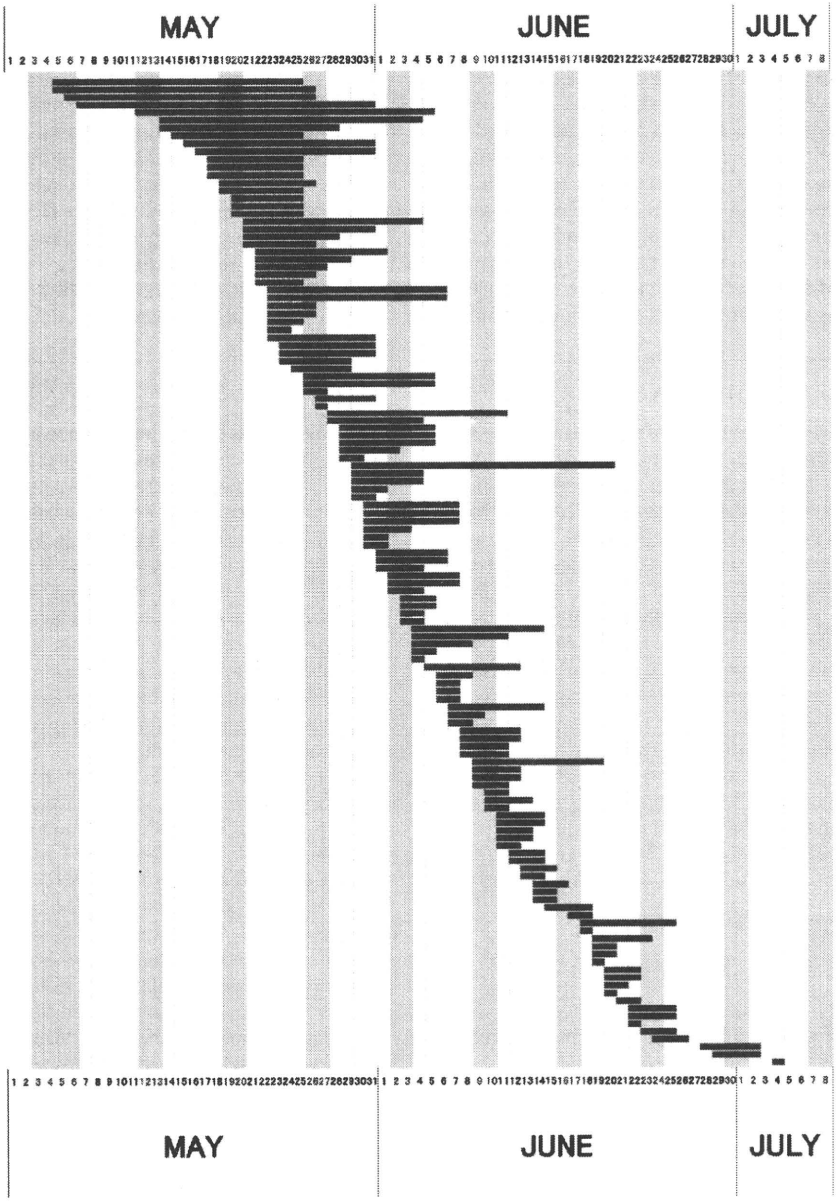

Fig. 1. Calendar showing duration of cough in patients during pertussis outbreak in a university. Patients (n = 128) with probable pertussis were asked for the date of cough onset during May to July 2007. Length of a bar shows the duration of cough until the date of serological analysis.

and were diagnosed with pertussis. A total of 9 students had a primary complaint of cough. These students shared a dining room and a kitchen in the same dormitory and frequently watched TV and played video-games together in the same room.

Cases in the club: In the club, a student complained of a paroxysmal cough for more than 2 weeks. Four students in the club subsequently complained of a persistent cough and were diagnosed with pertussis. A total of 8 students had a primary complaint of cough. All members of this club had dinner with the initial patient after a club activity, and also lived together in a training camp.

Symptoms of the patients: In most cases, the main symptom of pertussis has been reported to be a protracted cough (6,8). For the pertussis cases in this study, paroxysmal cough occurred in 53.9% of patients and nocturnal cough in 50.7% of patients (Table 1). Since some patients with a strong positive agglutinin titer were not conscious of their prolonged light cough, it is possible that patients with very mild symptoms transmitted pertussis to other people.

Serological tests: The bacterial agglutinin titers against the strain Yamaguchi of the outbreak group (median = 160×, upper quartile = 320×) were significantly higher than those of the control group (median = 40×, upper quartile = 160×) ($P < 0.001$) (Fig. 2A). Therefore, significantly more cases in the outbreak group had Yamaguchi agglutinin titers ≥160× (178/310, 57.4%) compared to the control group (74/246, 30.1%) ($P < 0.001$). In contrast, there was no significant difference in the Tohama agglutinin titers between the outbreak and control groups (median = 80×, upper quartile = 160×) (Fig. 2B).

To investigate the relevance of age, we compared the agglutinin titers in people ≤39 years old in the outbreak group (n = 282; 155 male, 127 female; age range 18–39 years; average age 21.2; median age 20) and in the healthy control group (n = 123; 65 male, 58 female; age range 21–39 years; average age 32.3; median age 32). The Yamaguchi agglutinin titers of the outbreak group (median = 160×, upper quartile = 320×) were significantly higher than those in the control group (median = 40×, upper quartile = 80×) ($P < 0.001$) (Fig. 3A). Therefore, significantly more cases in the outbreak group had Yamaguchi agglutinin titers ≥160× (165/282, 58.5%) compared to the control group (27/123, 22.0%) ($P < 0.001$). In contrast, there was no significant difference in Tohama agglutinin titers between the outbreak and control groups (median = 80×, upper quartile = 160×) (Fig. 3B).

Relationship between the Yamaguchi agglutinin titers and the duration of cough: We analyzed the relationship between the Yamaguchi agglutinin titers and the duration of cough in the students. Of the 93 student probable cases, 28 had a Yamaguchi agglutinin titer ≤80× and a cough for 3.8 ± 2.5 days, while 65 had a Yamaguchi agglutinin titer ≥160× and a cough for 11.1 ± 23.3 days. Therefore, the duration of cough was longer in student probable cases with Yamaguchi agglutinin titers ≥160× than in cases with Yamaguchi agglutinin titers ≤80× (Table 2A). Of the 17 students in the dormitory and the club, 7 had a Yamaguchi agglutinin titer ≤80× and a cough for 3.8 ± 6.0 days, while 10 had a Yamaguchi agglutinin titer ≥160× and a cough for 10.3 ± 6.3 days. Therefore, the duration of cough was longer in students in the dormitory and the club with Yamaguchi agglutinin titers ≥160× than in cases with Yamaguchi agglutinin titers ≤80× (Table 2B).

Genetic tests: Molecular diagnosis of *B. pertussis* infection was performed using the LAMP method (5). Of the 60 patients with suspected pertussis infection, 20 (33.3%) were positive by the LAMP assay, indicating that the outbreak was caused by *B. pertussis* infection. Interestingly, all positive

Table 1. Symptoms of the pertussis patients in this study

Symptom	%
Paroxysmal cough	53.9
Nocturnal cough	50.7
Whooping cough	14.7
Dyspnea	17.5
Post-tussive vomiting	6.3
Speaking induced cough	23.8

Probable cases (n = 63).

Table 2. Analysis of the relationship between the Yamaguchi agglutinin titer and duration of cough in students

(A) Students probable cases (n = 93)

Agglutinin titer	≤80×	≥160×
Duration of cough (d)	3.8 ± 2.5 (n = 28)	11.1 ± 23.3 (n = 65)

Result of Mann-Whitney U-test; P = 0.078.

(B) Students in the dormitory and the club (n = 17)

Agglutinin titer	≤80×	≥160×
Duration of cough (d)	3.8 ± 6.0 (n = 7)	10.3 ± 6.3 (n = 10)

Result of Mann-Whitney U-test; P = 0.012.

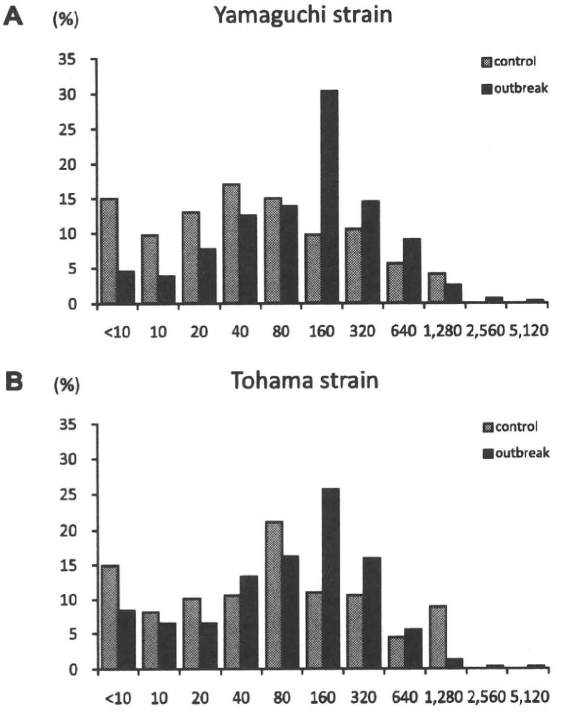

Fig. 2. *B. pertussis* agglutinin titers of people in the outbreak and control groups. (A) *B. pertussis* agglutinin titers against the strain Yamaguchi. (B) *B. pertussis* agglutinin titers against the strain Tohama. x axis, agglutinin titer. y axis, percentage of patients. ▨, control group (n = 246). ■, outbreak group (n = 310).

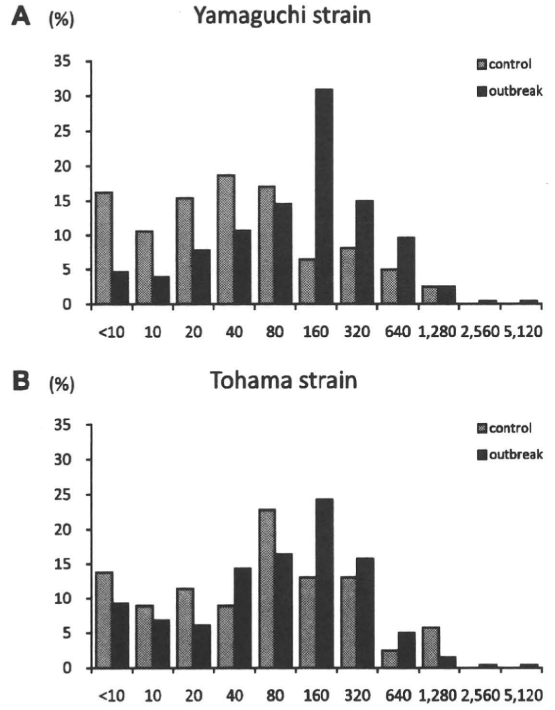

Fig. 3. *B. pertussis* agglutinin titers of people ≤39 years old in the outbreak and control groups. (A) *B. pertussis* agglutinin titers against the strain Yamaguchi. (B) *B. pertussis* agglutinin titers against the strain Tohama. *x* axis, agglutinin titer. *y* axis, percentage of patients. ▨, control group (n = 123). ■, outbreak group (n = 282).

samples had been collected from patients (n = 34) at an early stage (before June 4) of the outbreak (positive rate 20/34, 58.8%). No positive samples were collected at the late stage (after June 5). The difference in the number of positive samples between the early and late stages may have been due to antibiotic prophylaxis or therapy. In fact, no early-stage patients received antibiotics before the LAMP assay, whereas at least 6 of 26 late-stage patients had received antibiotics.

DISCUSSION

The index case of the pertussis outbreak in this study was probably a university student living in the dormitory. An infection in such a small, semi-closed population can spread like a wave throughout the population, leading to a mass outbreak. In the university, to control further spread of the pertussis outbreak, the cooperation of students and staff members who had complained of a persistent cough was necessary. Prescription of macrolides as chemoprophylaxis to people who had contact with pertussis cases in the dormitory, the club, or the office seemed to be effective in controlling the outbreak.

The 2007 pertussis outbreak suggests that a pertussis vaccination system should be reconsidered in Japan. The pertussis vaccine has been administered to 86% of pertussis patients (9,10). However, the duration of effectiveness of vaccination-induced pertussis immunity is 4–12 years (9,10). Attenuation of immunity is thought to be due to a decrease in the number of plasma cells producing specific antibodies and specific CD8+ cells, although the number of memory B and T cells does not decrease (9,10). Currently, in Japan, the DTaP vaccine for pertussis vaccination is administered to infants in 4 doses: 3 doses during an infant's first year and once at 1 year. In contrast, in the USA, DTaP vaccine is administered in 5 doses (at 2, 4, 6, and 15–18 months, and at 5 years), and an additional tetanus toxoid, reduced diphtheria toxoid and acellular pertussis (Tdap) vaccine dose is recommended at 11–18 years, for a total of 6 pertussis vaccine doses (11). It has been reported that Tdap vaccine prevents *B. pertussis* infection in adolescents and adults (12,13). Therefore, since there is a significant risk of pertussis outbreaks in adolescent and adult populations, pertussis vaccination of adolescents and adults should be considered in Japan.

In this study, we demonstrated that the bacterial agglutination assay against strain Yamaguchi might be a useful tool for diagnosis of adolescent and adult pertussis, especially in young adult patients (18–39 years old) when a cut-off value of ≥160× is used to determine positive agglutination results (Table 2A, B, Fig. 3A, B). The major agglutinogens of *B. pertussis* Tohama are agglutinogens 1, 2, and 4, while those of the Yamaguchi strain are agglutinogens 1, 3, and 6 (3,14, 15). In the outbreak group, the Yamaguchi agglutinin titers were significantly higher than in the control group, but the Tohama agglutinin titer was not statistically different between these two groups. This observation strongly suggests that there was an increase in the antibody against agglutinogens 3 and 6 in outbreak group patients, although *B. pertussis* could not be isolated.

It has been reported that anti-pertussis toxin (PT) IgG of 100–125 units/ml in a single serological test is diagnostic of a *B. pertussis* infection in the previous 2–3 weeks in Europe (16–19). Surprisingly, it has also been reported that a *B. pertussis* culture-positive infant was positive by the Yamaguchi agglutinin assay but negative for anti-PT IgG at 7 days after hospital admission (20). These observations indicate that the Yamaguchi agglutinin titer might not always be in agreement with that of anti-PT IgG. Therefore, both anti-PT IgG and Yamaguchi agglutinin titers should be measured to avoid an anti-PT IgG false-negative and to yield a more accurate diagnosis.

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Marked difference between adults and children in *Bordetella pertussis* DNA load in nasopharyngeal swabs

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Abstract

Bordetella pertussis is the aetiologic agent of whooping cough, a common cause of severe respiratory illness in children and prolonged mild cough in adults. To understand some of the reasons for differences in clinical symptoms between adults and children, we measured *B. pertussis* DNA loads in nasopharyngeal swabs (NPS) from 19 adults and 40 children (including 14 infants) by quantitative IS*481* real-time PCR. All cases had been pre-diagnosed with the *B. pertussis*-specific loop-mediated isothermal amplification method. The mean PCR threshold cycles for adult and child NPS were 34.9 and 27.1, respectively, indicating a significantly lower *B. pertussis* DNA load in adults than in children ($p < 0.001$). Moreover, adults had very low DNA loads during both early and later stages of the disease. When corresponding bacterial loads in NPS were calculated for *B. pertussis* Tohama cells using a standard curve, the mean number of bacterial cells taken with a rayon-tipped swab from an adult, older child and infant was estimated to be 320 (95% CI 120–910), 2.1×10^4 (95% CI 5.3×10^3 to 8.3×10^4) and 1.1×10^6 cells (95% CI 1.2×10^5 to 8.9×10^6), respectively. This indicates that the *B. pertussis* load in NPS is closely correlated with patient age. Our observations suggest that adult pertussis is characterized by a lower bacterial load in the nasopharynx, resulting in milder symptoms and negative cultures.

Keywords: Adults, bacterial load, *Bordetella pertussis*, children, nasopharyngeal swab, real-time PCR
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Introduction

Bordetella pertussis, a highly communicable gram-negative coccobacillus, is the aetiologic agent of whooping cough, which has been a major acute respiratory infection, resulting in severe childhood illness and infant death [1]. In Japan, the incidence of pertussis cases in adolescents and, especially, adults, has significantly increased from the early 2000s; this has also been seen in other countries with high vaccination coverage [2]. Adolescents and adults are assumed to be the primary reservoir of *B. pertussis* and play a crucial role in the transmission of the microbe to infants and unvaccinated children [3–5]. The clinical symptoms of pertussis in adolescents and adults vaccinated during childhood are different from typical presentations in children, and may consist only of a prolonged cough [6–9]. Moreover, culture of *B. pertussis* has a much lower sensitivity for diagnosis of pertussis in adults than in children [5,9]. Although *B. pertussis* infection is known to present differently in adults and children, the differences in the bacterial load in the nasopharynx remain unclear.

During the last decade, nucleic acid amplification tests have revolutionized the laboratory diagnosis of *B. pertussis* infections. Various detection methods, including real-time PCR and loop-mediated isothermal amplification (LAMP), have been developed that target different regions of *B. pertussis* genome [10–13]. Previously, we developed a LAMP assay method targeting the pertussis toxin promoter region, which provided rapid, sensitive and highly specific detection of *B. pertussis* DNA [11], although the amplification efficiency is too high to make a quantitative assay of the bacterial load in clinical specimens. By contrast, real-time PCR targeting insertion sequence IS*481* is a useful tool not only for rapid

and sensitive diagnosis, but also for quantitative analysis. The IS*481* real-time PCR can detect other *Bordetella* subspecies, such as *Bordetella holmesii*, *Bordetella bronchiseptica* and *Bordetella parapertussis*, although this assay is now widely used for the diagnosis of *B. pertussis* [14,15]. In neonates and young children with pertussis, the IS*481* real-time PCR showed that the *B. pertussis* DNA load in nasopharyngeal swabs (NPS) persists for a long time (3 weeks) after administration of antimicrobials [16,17]. For other pathogenic agents causing respiratory tract infections, such as *Moraxella catarrhalis* and *Streptcoccus pneumoniae*, the quantitative changes in bacterial DNA load in NPS correlate with the numbers of organisms detected by semiquantitative culture [18,19].

The present study aimed to determine *B. pertussis* DNA loads in NPS among adults and children, who were confirmed to have pertussis by *B. pertussis*-specific LAMP assay, by using quantitative IS*481* real-time PCR. We also investigated the relationship between *B. pertussis* load and bacterial genotypes.

Materials and Methods

Clinical samples

Approximately 200 NPS were obtained from adults (≥16 years old) and children (≤15 years old) with suspected pertussis between June 2007 and September 2009. The NPS were collected with sterilized rayon-tipped swabs (Eiken Chemical Co., Ltd, Tokyo, Japan) and then transported to the National Institute of Infectious Diseases, Japan. NPS were immersed in 0.5 mL of saline, vortexed, and precipitated by centrifugation (20 000 g for 10 min). Total DNA was extracted from the precipitation using QIAamp DNA Micro-kit (Qiagen, Hilden, Germany), and eluted with 25 μL of the AE elution buffer. The DNA samples were stored at −20°C until used.

LAMP assay

To confirm *B. pertussis* infection, a *B. pertussis*-specific LAMP assay was performed on DNA samples from NPS as described previously [11]. The LAMP amplification was performed with a 60-min reaction, and confirmed with real-time monitoring of the increase in turbidity using LA-320C (Eiken Chemical Co., Ltd). Among the DNA samples tested, 19 from adults and 40 from children (14 infants and 26 older children) were positive in the LAMP assay (≥0.1 turbidity), respectively. The vaccination history was obtained in 13 infants and 12 older children, but not in adults. Of 13 infants, 11 (85%) had never been immunized with pertussis vaccine. By contrast, ten (83%) of 12 older children had received four doses of pertussis vaccine.

IS*481* real-time PCR

Quantitative real-time PCR targeting IS*481* was performed by an Applied Biosystems 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) as described previously [12], with minor modifications. Briefly, the IS*481* real-time PCR amplifications were done in 20-μL reaction volumes containing 1× *Premix Ex Taq*™ (Perfect Real Time; Takara Bio Inc., Shiga, Japan), 0.9 μM primer PPert and APPert, 0.25 μM TaqMan probe SPert, 0.4 μL of 50× Rox reference dye II, and 2 μL of DNA sample. The PCR conditions were 15 s at 95°C, followed by 40 cycles of 95°C for 3 s and 57°C for 30 s. Real-time data were analyzed by Sequence Detection Systems software, version 1.4 (Applied Biosystems). Standard curve was generated with ten-fold serial dilutions of *B. pertussis* Tohama DNA from 10 ng (2.4×10^6 bacterial cells) to 0.1 fg (0.024 bacterial cells). The number of bacterial cells was calculated with the Tohama genome size of 4.1 Mbp (2.4 genomic copies/10 fg DNA) [20].

The sampling efficiency of NPS and the presence of PCR inhibitors were examined by amplification of the human β_2-microglobulin gene with primers B2M-TR-1 and B2M-TR-2 [16] using SYBR green-based real-time PCR. Each 12 adult and child DNA samples, which were randomly selected from LAMP-positive samples, showed β_2-microglobulin C_t values in the range 15.1–24.8 (mean 21.9) and 19.8–22.3 (mean 21.1), respectively, confirming that the respective sampling efficiency and PCR inhibition were almost identical between adult and child NPS samples.

Multilocus sequence typing (MLST)

B. pertussis allelic genes (pertussis toxin *ptxA*, pertactin *prn* and serotype 3 fimbriae *fim3*) were amplified directly from patient DNA samples by nested-PCR. The first PCR was performed in a 15-μL reaction volume containing 1.87 μL DNA sample, 0.44 mM concentrations of each dNTPs, 0.2 μM concentrations of each primer, and 0.3 U of KOD-FX DNA polymerase (Toyobo, Osaka, Japan). For the nested-PCR amplification, 1 μL of the first PCR product was added as the template to the PCR mixture containing the same components described above, except that the nested primers were used instead of the first set of primers (Table 1). Cycling conditions were: denaturation for 1 min at 94°C; 30 cycles of 10 s at 98°C, 30 s at 55°C, 45 s at 68°C; and a postextension of 5 min at 72°C. The PCR products were sequenced with the BigDye terminator v3.1 cycle sequencing kit on an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems). The combined allelic profiles were used to define MLSTs as described previously [2].

TABLE 1. Primers used in multilocus sequence typing analysis

Target gene	Primer name	Sequence (5'- to 3')	PCR	Coordinate*
ptxA	ptx-outerF	TAACGCGGGTCTATCACAAC	1st	3988790
	ptx-outerR	TAGAACGAATACGCGATGCT	1st	3989047
	ptx-innerF	GACCACGACCACGGAGTATT	2nd	3988824
	ptx-innerR	GTACACGAGAACCATCGCCT	2nd	3989021
prn	prn-AF	GCCAATGTCACGGTCCAA	1st	1098595
	prn-AR	GCAAGGTGATCGACAGGG	1st	1099163
	prn-innerF	GTCATTGCAGCCGGAAGACC	2nd	1098657
	prn-innerR	CCGGTCTCGATGACATTGCC	2nd	1099111
fim3	Fim3-FI	ATGTCCAAGTTTTCATACCC	1st	1647602
	Fim3-RI	GGTGACCTTGCCGGTAAA	1st	1648082
	fim3-innerF	CCAGCACCCTCAACCATATC	2nd	1647738
	fim3-innerR	GGCTTGCGTGGTTTTGTC	2nd	1648055

*Coordinates in *Bordetella pertussis* Tohama genome sequence NC02929.

Statistical analysis

Data were analyzed using the Mann–Whitney *U*-test. $p < 0.05$ was considered statistically significant.

Results

Sensitivity and specificity of IS*481* real-time PCR

The real-time PCR with ten-fold serial dilutions (0.1 fg to 10 ng) of *B. pertussis* Tohama DNA was able to detect bacterial DNA over a linear range between 10 fg (2.4 bacterial cells) and 10 ng (2.4×10^6 bacterial cells) per reaction mixture ($r^2 = 0.99$) (Fig. 1). On the basis of three independent experiments, the detection limit was a threshold cycle (C_t) of 37.6 ± 0.3, corresponding to 2.4 cells of the Tohama. The analytical sensitivity of the IS*481* real-time PCR was equal to that of the *B. pertussis*-specific LAMP assay [11].

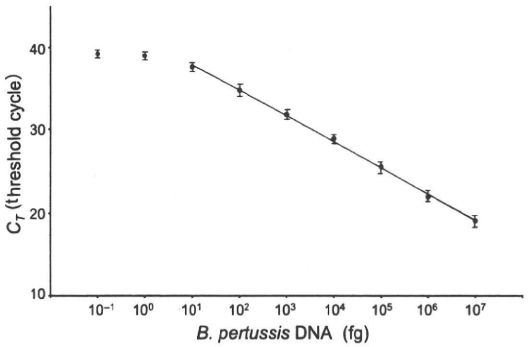

FIG. 1. Detection limit for linear calibration of IS*481* real-time PCR. Serial dilutions of *Bordetella pertussis* Tohama DNA were subjected to the real-time PCR. Data are the mean ± SD for three independent experiments.

Fifty-nine LAMP-positive (19 adults and 40 children) and 24 LAMP-negative DNA samples (six adults and 18 children, randomly selected) were subjected to the IS*481* real-time PCR. The LAMP-positive samples had C_t values in the range 14.6–39.5 (mean 29.7). By contrast, when the real-time PCR assay was applied to the LAMP-negative samples, the C_t values were in the range 37.2–40 (mean 39.7) (data not shown). Twenty (83%) of 24 LAMP-negative samples had a C_t value of 40 (i.e. no detectable amplification). The IS*481* real-time PCR and LAMP results showed a high level of agreement (77/83; 93%) with 55/83 found to be positive in both assays and 22/83 found to be negative in both assays, when a C_t value >37.6 was used as the cut-off for the real-time PCR.

B. pertussis DNA loads among children and adults

Figure 2a shows C_t values of LAMP-positive DNA samples from 40 children (mean age 6.0 years; age range 0–15 years) and 19 adults (mean age 43.3 years; age range 22–83 years). The child samples had C_t values in the range 14.6–39.5 (mean

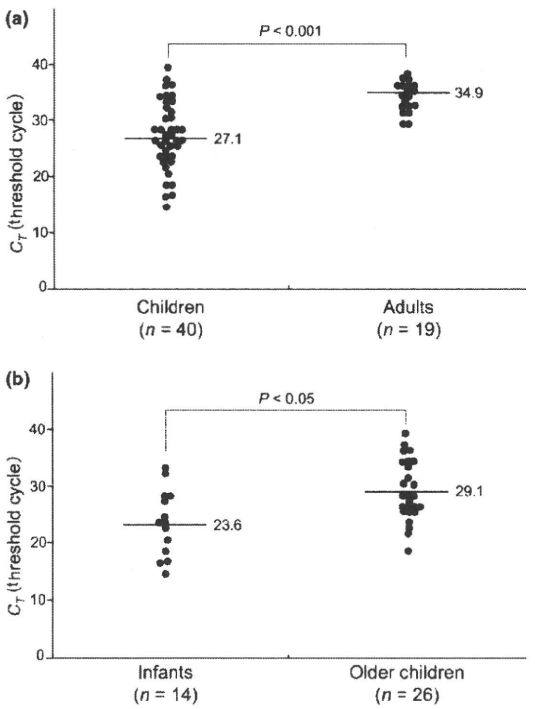

FIG. 2. Comparison of *Bordetella pertussis* DNA loads in nasopharyngeal swabs among children and adults with pertussis. (a) Children (infant and older children, $n = 40$) versus adults ($n = 19$). (b) Infants ($n = 14$) versus older children ($n = 26$). The DNA samples (2 μL) were subjected to IS*481* real-time PCR. Horizontal bars indicate mean C_t values.

27.1), whereas the adult samples had significantly higher C_t values in the range 29.1–39.0 (mean 34.9) within a narrow range. Statistical significance was observed with respect to the C_t values between children and adults ($p < 0.001$). Figure 2b also shows C_t values of LAMP-positive DNA samples from 14 infants (mean age 2.9 months; age range 1–6 months) and 26 older children (mean age 9.2 years; age range 2–15 years). The infant samples had C_t values in the range 14.6–33.8 (mean 23.6). By contrast, the older child samples had higher C_t values in the range 18.8–39.5 (mean 29.1) (p 0.011).

The clinical information about cough duration was obtained from 16 adult patients. Eight adults had a cough duration of less than 14 days (mean 8.6 days) at the time of sampling, and other adults had a duration of more than 15 days (mean 30.3 days). The C_t values (mean C_t value of ≤14 days, 35.9; >15 days, 34.8) were not statistically significant between these two groups (p 0.92) (data not shown).

Relationship of *B. pertussis* DNA loads and bacterial genotypes

On the basis of the MLST analysis, Japanese *B. pertussis* isolates could be classified into five genotypes (MLST-1 to MLST-5), as described previously [2]. Among 40 children NPS samples, the MLSTs in 33 samples were identified as: 17 MLST-1 (harboring *ptxA2*, *prn1* and *fim3A* alleles); 14 MLST-2 (*ptxA1*, *prn2* and *fim3A*); and two MLST-4 (*ptxA1*, *prn2* and *fim3B*). The mean C_t values for MLST-1, -2, and -4 were 27.0, 24.8, and 28.6, respectively. Comparison of C_t values for MLST-1 and -2 revealed no statistically significant difference (p 0.27) (data not shown). Among the infants and older children, no correlation was found between *B. pertussis* DNA loads and the bacterial genotypes.

Discussion

To our knowledge, this is the first report of a precise comparative analysis of *B. pertussis* loads in adults and children. The results obtained clearly indicate that adults had very low *B. pertussis* DNA loads in their NPS compared to children, especially infants. When bacterial loads in NPS were calculated for *B. pertussis* Tohama cells using a standard curve, the mean numbers of bacterial cells taken with a rayon-tipped swab from adults, older children and infants were estimated to be 320 (95% CI 120–910), 2.1×10^4 (95% CI 5.3×10^3 to 8.3×10^4) and 1.1×10^6 cells (95% CI 1.2×10^5 to 8.9×10^6), respectively (Table 2). Surprisingly, the bacterial numbers in adults were 340-fold and 65-fold lower than those in infants and older children, respectively. In general, adults with pertussis showed only prolonged cough illness and had less typical symptoms than children [3,6–9]. In addition, vaccinated asymptomatic children had significantly fewer *B. pertussis* than symptomatic patients [21]. Our experimental observations strongly suggest that the lower nasopharyngeal bacterial load in adults is related to the atypical and milder symptoms in adult pertussis.

TABLE 2. Number of *Bordetella pertussis* cells taken with a rayon-tipped swab from infant, child and adult patient

Patient	Number of patients	Mean age (range)	*B. pertussis* cells/swab (95% CI)[a]
Infant	14	2.9 months (1–6 months)	1.1×10^6 (1.2×10^5 to 8.9×10^6)
Older child	26	9.2 years (2–15 years)	2.1×10^4 (5.3×10^3 to 8.3×10^4)
Adult	19	43.3 years (22–83 years)	320 (120–910)

[a]Bacterial cells were calculated for *B. pertussis* Tohama cell.

Previously, Bidet *et al.* [16] demonstrated that *B. pertussis* DNA loads in NPS decreased progressively during antibiotic treatment in children. Several factors are also considered to affect *B. pertussis* load in the human nasopharynx, including patient age, previous vaccination or infection, and host genetic background. In the present study, infants had a larger *B. pertussis* DNA load than older children, and most (85%) of the infants had not received pertussis vaccination, whereas most (83%) of the older children had received four doses of the vaccine. Our findings support the hypothesis that the *B. pertussis* DNA load is affected by vaccination. Unfortunately, this does not apply to adults because pertussis vaccines are unable to provide lifelong immunity [22]. The duration of immunity post-vaccination is estimated to be in the range 4–12 years and, therefore, the bacterial loads in adults may be affected by other factor(s) besides the vaccination.

B. pertussis culture has been taken as a gold standard diagnostic method because it is highly specific. However, the culture has limited sensitivity for previously vaccinated persons, especially adolescents and adults [5,8,9]. In the present study, we demonstrated that adults with pertussis had very low *B. pertussis* DNA loads in their NPS during both early and later stages of the cough illness. This finding suggests that the diagnosis of pertussis by bacterial culture is difficult in adults even if NPS are obtained in the early stage of the illness. Compared with the culture method, nucleic acid amplification tests such as IS*481*-based real-time PCR and *B. pertussis*-specific LAMP provide a highly sensitive procedure for detection of *B. pertussis* DNA [11,13,14]. On the basis of our findings, we recommend the performance of

nucleic acid amplification tests for an accurate diagnosis of pertussis, especially when adult pertussis cases are suspected.

During the last three decades, genetic divergence in *B. pertussis* has been observed in many countries [23–27]. In Japan, the circulating strains have shifted mainly from MLST-1 to MLST-2. The MLST-1 strains include vaccine-types *ptxA2* and *prn1* alleles, whereas the MLST-2 strains include nonvaccine-types *ptxA1* and *prn2* alleles [2]. This genetic shift has been speculated to have resulted from adaptation of the bacterial population to vaccine-induced immunity [23,24,28,29]. The bacterial load of the emerging MLST-2 strains in the nasopharynx has not previously been investigated. We therefore examined the bacterial DNA loads of MLST-2 and MLST-1 strains but found no association between the DNA loads and their genotypes among infants and older children. This would suggest that emerging MLST-2 strains have the ability to produce similar bacterial loads to those of the the MLST-1 strains.

In conclusion, the *B. pertussis* DNA load in NPS depends highly on patient age. Adults had very low *B. pertussis* DNA loads and organism numbers in their NPS during both the early and later stages of the cough illness, and this explains the culture-negative results in adult pertussis cases. To make an accurate diagnosis of adult pertussis, nucleic amplification tests such as the *IS481* real-time PCR and *B. pertussis*-specific LAMP assays are recommended as sensitive methods.

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Transparency Declaration

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食品衛生研究

Vol.60, No.3, 2010

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(表紙；高橋真紀)

食中毒の疫学研修講座 18

食中毒の「原因」と回収問題

Training Program on Food-borne Disease Epidemiology 18
"Cause" of Food-borne Disease and Recall

岡山食中毒の疫学研修プログラム研究会
岡山大学大学院環境学研究科　津田　敏秀
岡山市保健所　槌田　浩明，中瀬　克己，溝口　嘉範
岡山理科大学総合情報学部情報科学科　山本　英二
岡山大学大学院医歯薬学総合研究科　土居　弘幸，土橋　酉紀，頼藤　貴志
鈴木　越治，鹿嶋小緒里

ポイント

1．食中毒の「原因」についてきちんと整理する。
2．食中毒患者数と曝露有症者の関係を整理する。
3．病因物質は対策の必要条件ではない。
4．大事件のときには，大学研究者や医療従事者に調査を任せっきりにしたり，対策を講じる(処分を行う)ことを躊躇したり，対策をとらない理由を探してしまったりすることがあるが，これらは避けるべきである。
5．回収命令および自主回収は，被害拡大予防の役に立つように，十分に整理しておかなければならない。

Ⅰ　食中毒の原因とは

食中毒疫学調査を何のためにするかというと，原因を明らかにして対策を講じ，わかりやすい報告書を書いて，将来の予防に役立てるためである。原因とはこの場合，原因食品と原因施設である。ところで，原因に関して共通の認識を一応作っておく必要があるので，ここで簡潔に述べておく。

スコットランドの哲学者であるディビッド・ヒュームは，原因を次のように定義づけている。「われわれは，別な事象に伴われるある事象(an object followed by another)を原因と定義しよう。ここで，2番目の事象は，最初の事象が起こらなかったとしたら，決して起きることがなかったであろうものである」[1,2]。つまり，原因とは，結果が後に引き続くものである。ただし，その際，もしその原因が起きなければその結果が決して起きなかったであろうということが必要であるというのだ。

これを食中毒事件でいうと，原因食品とは，それを喫食した後に食中毒関連症状(下痢など)が引

表1　喫食しなかった人は喫食者が喫食しなかった場合の置き換え

	喫食した人	左の人たちが喫食しなかった場合	喫食しなかった人
発　症	a人	a'人	b人
非発症	c人	c'人	d人
合計	a＋c人	a'＋c'人	b＋d人

置き換え：このズレを交絡と見ることができる

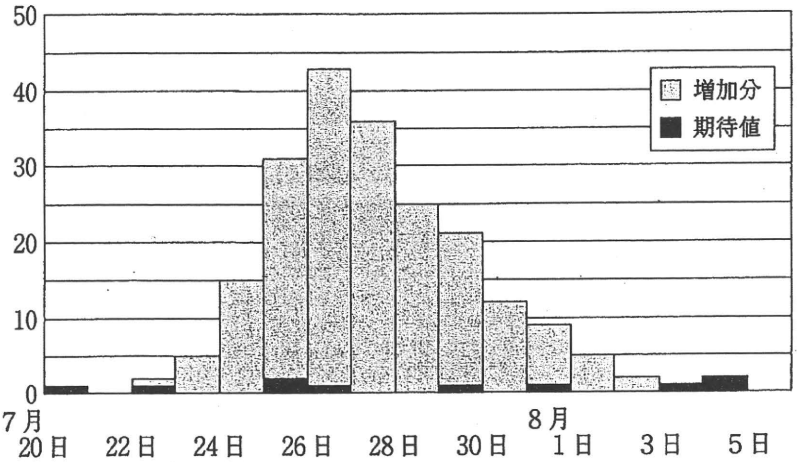
き続くものであり，それを喫食しなければその下痢などの食中毒関連症状が決して現れなかったであろう食品をいう。原因施設は，そこを利用した後に食中毒関連症状(下痢など)が引き続くものであり，そこを利用しなければその下痢などの食中毒関連症状が決して起きなかったであろう施設をいう。しかし，原因食品を喫食してその後に発症している個人において，「原因食品を喫食しなければ発症しなかったかどうか」など確認しようがない。これが，因果関係が「難しい」といわれる根源的な部分である。しかし，難しいのではなく，現実には観察不可能なので判断のしようがなく，したがって合意できないのである。

われわれは，これを因果関係の原因自体は観察可能で結果自体も観察可能だが，因果関係自体は目にも見えず，音も聞こえず，触ることもできず，観察不可能であると説明する。ヒュームは「必然性は心のなかに存在する何ものかであって，対象のなかにあるのではない。もし必然性を物体のなかにある性質と考えるなら，必然性のほんのかすかな観念をまったくもたないか，それとも必然性は原因から結果へ，もしくは結果から原因へと，経験された結びつきにしたがって移る思考の規定にほかならないか，そのいずれかである」と説明し，因果関係が心の結合慣習から作られる観念にすぎないと主張した。これでは，客観的因果律の存在が否定も肯定もできないことになる。原因と結果は経験的に認められるが，因果法則などは観念論として否定することにつながる[3]。

しかし問題なのは，因果関係を判断しなければ，現実社会に生きるわれわれの日常生活は成り立たないことである。われわれはなんらかの因果関係が存在すると思って生活しているし，そのことは誰もが認めるところである。現実的に観察不可能だからといって，ヒュームの問題をそのままにしておくわけにはいかない。

そこで，われわれは因果判断の根拠を求めて，原因食品を喫食していない個人を観察することになる。このような観察は実現可能なものの，個人では置き換えの際のズレが激しすぎることが予想できる。統計学的にいうとバラツキが大きすぎるのである。そこで複数の人数を観察することになる。

まず，原因食品を喫食した人びとを観察して発症者数を数える。そして，その人たちが仮に喫食しなかったときの発症者数と比較することは不可能なので，代わりに，実際に喫食しなかった人びとを観察して発症者数を数え，原因食品を喫食した人びとにおける発症者数と比較する。これで2×2表が完成する(表1)。したがって，2×2表は「原因食品を喫食した人」，「原因食品を喫食した人が喫食しなかった場合」の後者を単に「原因食品を喫食しなかった人」に置き換えているのであ



アウトブレイクが発生したとき(増加分＋期待値)とアウトブレイクがないとき(期待値)の患者発生数がどうなったのかを示す

図1　仮想事例の流行曲線

る。この置き換えの際の「原因食品を喫食した人が喫食しなかった場合」と「原因食品を喫食しなかった人」とのズレによる誤差を交絡とするのが，交絡要因の厳密な定義である。

症例対照研究も，症例における喫食歴の割合とその人たちが仮に症例が発症しなかったときの喫食歴の割合を比較したいのであるが，後者の割合は観察できないので，代わりに非発症者である対照の喫食歴と比較している。

このように整理して考えてみると，実際に起こった事件での対象者のデータを直接的に用いて，疫学は，厳密にかつ論理的に因果関係を検証していることがわかる。疫学調査を通じて，その事件の原因食品と原因施設を，その事件のデータで究明しなければならないことも理解できる。その事件の原因食品は，回収命令の対象食品を決める際に判明させなければならないし，その事件の原因施設は，営業停止・禁止の対象を決める際に判明させなければならないのだ。

真実は喫食調査票と症状調査票にあるといえる。だからこそ，真実が2×2表に書き記されるように喫食調査と症状調査を行うのである。

Ⅱ　食中毒患者数

食中毒事件において，食中毒患者のカウントは，原因食品を喫食し関連症状を発症した人びと，もしくは原因施設を利用し関連症状を発症した人びとをカウントすることによって行われる。しかし，この原因施設を利用し関連症状を発症した人びとのなかには，仮に原因施設を利用しなかったとしても関連症状を発症する因子をもともとそろえていた人びと(期待値)を少数含んでしまっている(図1)。しかし，仮に原因施設を利用しなかったら症状を発症しなかった人びとと，仮に原因施設を利用しなかったとしても関連症状を発症する運命にあった人びとを区別するのは不可能なので，食中毒事件における食中毒患者数は原因施設を利用し関連症状を発症した人びとをカウントすることになる。

原因食品を喫食して発症した人のなかには，仮に原因食品を喫食しなくても発症した運命の人が，少数含まれている。しかし，この人たちの呈する食中毒関連症状と，曝露により発症した人たちの呈する食中毒関連症状は，症状としてはまっ

たく区別がつかないことがほとんどである。これを，模式的に仮想事例の流行曲線で示すと，図1のようなヒストグラムが描ける。増加分が，曝露によって発症した患者をカウントした部分で，期待値が，曝露がなくても発症したであろう患者をカウントした部分である。

しかし，症状ではまったく区別がつかないので誰もこの増加分か期待値かを区別することはできないし，通常のアウトブレイクでは増加分に比べて期待値の部分が無視できるほど小さいと考えられる。だからこそアウトブレイクとして認識されるのだ。このことは期待値の部分が大きい状況を想像してみればわかる。したがって，通常は，症状が曝露に対して非特異的であるにもかかわらず，曝露され発症した人全員を食中毒患者として認識し，食中毒患者数をカウントしている。

加えて，原因食品がすべて病因物質により汚染されているような事例はむしろ少なく(確認されることはほとんどないが)，同じ事例で提供された原因食品でも病因物質に汚染されているものと汚染されていないものが存在することになる。しかし，原因食品のうち汚染されていない食品を喫食しても発症したか否かは確認しようがないので，同じ食中毒患者数としてカウントする。期待値が増加分に比べて無視できるほど小さいので可能である。また，原因施設を利用しても原因食品を喫食していない人も存在するはずだが，この人たちが原因食品を喫食したか否かに関して，記憶間違いのしようがない場合を除いて，通常は原因施設を利用し関連症状を発症した人を食中毒患者数としてカウントしている。

このような問題を吟味すると，結局食中毒事件の探知とは，通常の発症者数の期待値を上回る患者を探知することだという点がよくわかる。この

期待値は，時間的な期待値，場所的・空間的な期待値，人の要因間での期待値などが計算できる。時間的な期待値は，前年同時期の疾病発生数などで求めることが可能になる。場所的・空間的な期待値は，全国あるいは地域全体，もしくは隣接した地域の疾病の発生数や発生率で求めることが可能である(発生数は分母をそろえる必要がある)。例えば人の期待値は，職業別での一般的分布を期待値とする。特殊な職業において有症者が増加すれば，その職業に関して調査を進めることになる。

では，アウトブレイクの探知をできるだけ早く行うには，いったい何人の発症で，われわれは疾患のアウトブレイクを疑うべきであろうか。CDC(米国疾病管理予防センター)では，同一集団で2名以上(要するに複数)の発症の場合からアウトブレイクとして考えるべきであるとしている。ただし，ボツリヌス症のような死亡などの危険性の高い食中毒や感染症の場合は，1名でもアウトブレイクとするべきとしている。

Ⅲ　曝露寄与危険度割合

原因施設を利用し関連症状を発症した人びと(食中毒患者数)のうち，何%が原因施設を利用したことにより関連症状を発症した人びとであったのか(いわば真の食中毒患者の割合)ということを推定する方法がある。曝露寄与危険度割合(過剰分画，原因確率，病因分画，必要確率とも呼ばれる)である。曝露して発症した人のなかには，曝露しなくても発症する運命だった人も混入している可能性を，定量的に見積もるためである。これも，非曝露群(原因施設を利用しなかった人びと)の発症割合もしくは相対危険度を利用して求めることになる。

原因施設を利用した人びとの数をN_1人とする。このなかで関連症状を発症した人びとの数は曝露群の発症割合の分子で，これをA_1人とする。原因施設を利用した人の発症割合は$A_1 \div N_1$で求められる。原因施設を利用した人びとN_1人が，仮に原因施設を利用しなかった場合の発症数をA_0人とすると，原因施設を利用した人がもし原因施設を利用しなかった場合の発症割合は，$A_0 \div N_1$で求められる。ちなみに曝露寄与危険度割合は，原因施設を利用し関連症状を発症した人数(食中毒患者数)のうち，真に原因施設の利用により関連症状を発症した人数の割合なので，

曝露寄与危険度割合$= (A_1 - A_0)/A_1$

となる。右辺の分母分子をN_1で割ると，

曝露寄与危険度割合$=$

$\{(A_1/N_1) - (A_0/N_1)\}/(A_1/N_1)$

A_0/N_1は，推定不可能なので，非曝露群の発症割合R_0で置き換えると，

曝露寄与危険度割合$= (R_1 - R_0)/R_1$

R_1：曝露群の発症割合，ただし，$R_1 = A_1/N_1$

R_0：非曝露群の発症割合

右辺の分母分子を，R_0で割ると，

曝露寄与危険度割合$= (RR - 1)/RR$

RR：相対危険度，ただし；$RR = R_1/R_0$

以上のように，曝露寄与危険度割合は，曝露レベルと症状の相対危険度から計算できる。曝露寄与危険度割合は，保健医療や法廷の場で用いられることが多い[4]。食中毒処理要領に基づくと法廷に資料を提出する場合もあり得るので，参考のために記した。曝露寄与危険度割合を巡る問題点，さまざまな議論や解説は，専門書をお読みいただきたい[5,6]。

Ⅳ　病因物質は対策の必要条件ではない

食品衛生法が施行されて間もない1949年に，静岡県浜名湖周辺で，浜名湖で採れたアサリ貝を周辺住民が拾って食べたために発生したアサリ貝を原因食品とする食中毒事件は，浜名湖アサリ貝

事件として知られる。この事件においては，病因物質が不明のまま食品衛生法が適用され，適用前に比べて傷病死の予防に大きな効果を上げ，その後の食品衛生法の適用の際に参考とされることが多い。

このような事例を待つまでもなく，病因物質は対策実施の必要条件ではない。その理由としては，

① 原因食品がわかれば回収命令，原因施設がわかれば営業停止・禁止というように，病因物質の判明は何らかの対策にもともと対応していない。

② われわれは日常生活において原因をミクロの世界のものとは考えていない。つまり汚染されたアサリ貝を食べたことが発症の原因で，これは「汚染物質が何か」より優先される。原因を絶つには，浜名湖産アサリ貝の喫食を止めればよいからである。

③ 病因物質の判明はこだわりだしたらきりがなく，時が経ち科学が発達するにしたがってさまざまなことが判明してくるので，科学が発達すればするほど対策が遅れるという矛盾が生じてしまう。

④ 新しく出てきた化学物質や新興感染症による食中毒症の場合には，病因物質の判明を待っていたら対策を半永久的に打てなくなる。

⑤ もともと日常的に，病因物質が判明する前に食品衛生法に基づく営業停止処分を行っている。

などが挙げられる。

それにもかかわらず，食品から病因物質が検出されることを待ってしまう事例が後を絶たない。対策を急ぐ必要がない場合には，これでも構わないが，対策を急ぐ場合には病因物質の究明を待っている間に被害はどんどん広がってしまうことになる。病因物質は対策の必要条件ではないことを忘れてしまっているのだ。

食中毒事件の原因究明を大学に任せっきりにすると，大学研究者は病因物質の究明にこだわる傾向があるので，行政によって通常なされる対策が著しく遅れてしまうことには注意しなければならない。1955年夏の西日本での森永ヒ素ミルク中毒事件では，岡山大学医学部小児科の教授が病因物質を検索し続け，乳児の症状が食中毒症であることがわかっているのに届出が数週間遅れた[7]。1968年の北九州を中心としたカネミ油症事件では，原因食品が最初の新聞報道で十分に判明しているのに，その新聞報道の３日後に，「油症研究班」が九州大学で立ち上げられた。この研究班は，研究班名に原因食品名が明記されているのに（油症の「油」は原因食品であるカネミ油の油），「原因究明」として病因物質の追求を目的に研究した。この間，営業停止は大幅に遅れ，回収命令はついに出されずに被害が広がった。大学医学部は，通常は食中毒対策を何も知らないと考えるべきである。

通常の病因物質である細菌でさえ，分離同定するには時間が多少かかるので，普段の料理店や仕出屋の集団食中毒事件では，食品衛生法に基づく営業停止の際に，病因物質の同定を待ったりはしない。しかしマスコミの注目が集まる大規模食中毒事件や大手企業の出した食中毒事件の際には，躊躇してしまい，原因食品もしくは原因施設が判明しているのにもかかわらず，食品衛生法を適用するのを先延ばしし，とりあえず病因物質の判明を待ってしまう傾向が見られる。これは，論理性を超えた感情が，因果判断に混入しやすいことを指している。

V　対策をとらないための理由探し

すでに説明してきたように，曝露があったことは確認でき，症状があったことも確認できる一方，曝露と症状の間に因果関係があるか否かは目