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A Legionellosis Case Due to Contaminated Spa Water and Confirmed by Genomic Identification in Taiwan

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***Abstract*: Tracing the source of a legionellosis (LG) case revealed that the *Legionella pneumophila* (LP) strain isolated from patient's sputum shared the same serogroup (SG) and PFGE-type with 4 LP strains obtained from a spa center. With a high LP-contamination rate (81.2%, 13/16) in all of its 16 basins, this spa center was also found to have a multi-genotypic distribution among its 13 LP isolates, which can be categorized into 5 PFGE-types. Despite such a serious contamination in the spa center, which usually had ca. 100 visitors per day, this male patient, bearing LG-risk factors of long-term heavy smoking and alcoholism, was the only case identifiable after an active investigation. To explore the possible reason for this sporadic infection, all 5 PFGE-types of LP isolated were assayed for their presence of two important virulent genes (*lvh* and *rtx*A) and were identified as either less-virulent (*lvh*$^+$, *rtx*A$^-$) or non-virulent (*lvh*$^-$, *rtx*A$^-$) types. The strong virulent type (*lvh*$^+$, *rtx*A$^+$) usually seen in clinical strains elsewhere was not found here. Moreover, the LG-causative type in this infection was the only one to be classified as the less-virulent type, with the presence of *lvh* gene indicating its relatively more virulent potential than other 4 PFGE-types. Accordingly, mutual interaction between LP's virulent potential and patient's health-status was suggested to be the force directing the opportunistic infection of this sporadic case. This is the first spa-associated infection caused by SG 2 of LP.**

***Key words*: *Legionella*, PFGE-type, Serogroup, Virulent potential**

Legionellosis (LG, infection by members of the genus *Legionella*) can range from mild respiratory illness to acute life-threatening pneumonia. The majority of LG cases are caused by *Legionella pneumophila* (LP), particularly serogroup 1 (18). Since the first outbreak in Philadelphia in 1976 (12), LP has been recognized as an important etiological agent of hospital- and community-acquired pneumonia. This microbe can survive in a wide range of temperature (5–65 C) and pH (5.5–9.5), particularly in warm and damp environments of 35–45 C which is their favorable growth temperature range. Because of their high survival rate in a thermal and wet environment, which happens to be the atmosphere regularly established in a whirlpool spa, numerous outbreaks of LG have been traced to the spa water as the source of their causative agents (2, 17, 20, 21, 23, 31).

Tracing the source of LG was often determined by linking environmental isolates to clinical isolates by various molecular subtyping methods, of which at least 7 kinds have been reported (15). Among them, amplified fragment length polymorphism (AFLP) and pulse-field gel electrophoresis (PFGE) were two methods most often used and highly recommended (3, 13). However, since serogroup variations were observed

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Abbreviations: AFLP, amplified fragment length polymorphism; CDC, Center for Disease Control; DFA test, direct fluorescence antibody test; IFA test, indirect fluorescence antibody test; LG, legionellosis; LP, *Legionella pneumophila*; *lvh*, *L. pneumophila* type IV secretion system genes; PCR, polymerase chain reaction; PFGE, pulse-field gel electrophoresis; *rtx*A, repeats in structural toxin gene A; SG, serogroup; TE buffer, buffer contains Tris-HCl and EDTA; UPGMA, the unweighted pair group method with averages; UV, ultra-violet ray.

from LP strains shared with identical molecular fingerprinting patterns, it was thus suggested that, in epidemiological investigation, genetic fingerprinting should be used in conjunction with serogrouping to avoid possible bias in data interpretation (3).

With the aid of molecular subtyping, it has become clear that LG-associated spa water often co-exists with polymorphous LP strains (i.e., multi-genetic types or multi-serogroups), despite usually only one of them being detected as the causative pathogen in the correspondent outbreak (2, 11, 17, 23). This natural screening of a virulent (clinical) strain from the other less- or non-virulent ones through infection had provided precious materials for studying the virulent potential of LP. Recently, by comparing the phenotypic and genotypic differences between clinical and environmental LP strains, the genetic basis for virulence differences among LP strains had been attributed to the presence or absence of certain virulence genes (15, 26, 27). Particularly, two of these genes, *lvh* and *rtx*A, were even discovered to have a strong association with LG. Strains having both genes were shown to be more virulent than strains that had either *lvh* and *rtx*A alone, while those, lacking both genes, were suggested not able to cause disease in humans (15).

In this report, we present the first spa-acquired LG case in Taiwan, which was confirmed by serogroup and PFGE-typing assays. Further analysis of the presence or absence of *lvh* and *rtx*A genes in all PFGE-types of LP isolates to try to explore their possible role for the first time in a spa-associated LG case was also included in this study.

Materials and Methods

Case finding. On February 14, 2005, a 39-year-old male developed an upper respiratory tract infection and was admitted to hospital on February 18. The patient's clinical condition comprised a fever of 39.9 C, arthralgis, dyspnea, cough, chill, and pneumonia. The patient had a long-term habit of heavy smoking and alcoholism but denied any drug abuse and travel history. The attending physician suspected that the patient might have contracted LG and notified Taiwan's CDC of this potential LG case on February 20, 2005.

Case definition. LG was diagnosed and confirmed by both laboratory and clinical diagnosis according to the guidelines issued by CDC in U.S.A. (5), in which three assays (i.e., culture identification, paired-serum antibody test, and urine-antigen detection) were used as the criteria for laboratory diagnosis, while checking for the appearance of typical LG symptoms (i.e., fever, cough, and pneumonia) was as the basis of clinical diagnosis.

Searching for additional cases. To search for additional cases, a standardized interview was employed to obtain information regarding the following lifestyle features of the patient in the 2 weeks preceding illness onset: health; work status and location; modes of transportation; time spent at home; recent travel history; home water supply; frequency of showering; and, visiting local businesses, local factories with cooling towers, and other areas. Based on the information obtained, a control group including the patient's family members, colleagues, and spa attendants were selected and surveyed for the onset of illness. In addition, all local health services and hospitals in Taipei city, where this spa center is located, were asked to report cases of pneumonia in persons who might have visited the spa center from February 1 until April 15, 2005.

Environmental investigation. Environmental investigation focused on water contamination. Water samples were collected in sterile 1-liter polypropylene bottles from (a) restroom tap faucets at local factories, (b) tap water faucets at the patient's home restroom, and (c) the public whirlpool spa basin before decontamination.

Laboratory diagnosis. Culture identification and enumeration of LP (cfu/ml) in water: Sputum was plated on both selective and non-selective buffered charcoal yeast extract agar (BCYE agar, BBL) following the procedure described in Murray et al. (22). Suspect colonies were chosen after 3–5 days of incubation and identified by biochemical characteristics. To serotype the colony, a direct fluorescence antibody (DFA) test was performed using a colony-coated slide and FITC-reagent (Zeus Scientific, Inc.). For enumeration of LP (cfu/ml) in water, the procedure as described in Den Boer et al. (11) was followed. Briefly, 1 liter of the water sample was concentrated by membrane filtration (0.2 μm) and the filtered residue was resuspended in 1 ml of sterile water. Of this suspension, 100 μl samples were heated 30 min at 50 C and, then, cultured without dilution and after 10- and 100-fold dilutions on BCYE agar supplemented with α-ketoglutarate and the *Legionella* MWY Selective Supplement SR118 (Oxoid, Ltd., Hampshire, England). Plate counts were done after incubation at 37 C with increased humidity for 5 days.

Paired-serum antibody test: Paired-acute and convalescent-phase serum were evaluated to determine whether there was a 4-fold or greater increase in the antibody titer against LP serogroup 1–6 by indirect fluorescent antibody (IFA) test. The antigen-coated slides and reagents for the IFA test (group 1–6) were purchased from Zeus Scientific, Inc.

Urine antigen detection: An enzyme-linked

immunosorbent assay kit (Zeus Scientific, Inc.) was employed in the detection of LP antigen in urine.

Genomic investigation. PFGE analysis was done as described in Pruckler (25), with slight modifications. Briefly, cultures grown for 72 hr on buffered charcoal-yeast extract agar were suspended in TE buffer (10 mM Tris-HCl [pH 8.0], 1 mM EDTA [pH 8.0]) with turbidity adjusted to 25 (Vitek special DR100 colorimeter). The plugs, after being digested overnight at 50 C with 10 U of *Sfi*I (New England Biolabs), were loaded into a 1% PFGE-certified agarose gel and run in 0.5% Tris-borate-EDTA buffer using a contour-clamped homogenous electric field system (Biometra) at 13 C and 200 V with increasing switch times from 2 to 40 sec for 25 hr. Gels were stained with ethidium bromide (0.5 mg/ml) for 30 min, washed with water for 30 min, and then photographed with UV illumination. Pattern clustering on a matrix of Dice coefficient was based on the unweighted pair group method with averages (UPGMA), with a band position tolerance=2% and an optimization setting=0.5%. Interpretations of PFGE results were based on banding pattern differences as suggested by Tenover et al. (32). Strains differing in ≤3 fragments were deemed only clonally related and described as subtypes of a given clonal type.

Virulence genes detection using PCR. The six pairs of primers and PCR conditions used in this study to detect *lvh* and *rtx*A genes were the same as those described in Samrakandi et al. (26).

Results

Case Definition

An isolate from patient's sputum was identified as *L. pneumophila* (LP), which was subsequently diagnosed as serogroup 2 in a DFA test. In addition, a 4-fold increase in *Legionella* antibody titers, from 256 to 4,096, was detected between the patient's paired acute- and convalescent-phase sera. These laboratory evidences, along with the clinical symptoms (i.e., fever, cough, and pneumonia), coincided with CDC's criteria (U.S.A.), confirming this LG case despite the urine-antigen detection testing negative.

Searching for Additional Cases

Information obtained from the standardized interview revealed that the known medical risk factors for this patient were long-term heavy smoking and alcoholism, and that this patient often used a whirlpool spa at a public center. Thus, a control group of 144 members including the patient's family, colleagues and spa attendees, was selected and surveyed for the retrospect and prospective onset of illness. However, none of them was found to have or to develop LG by the end of this investigation on April 15. Replies from local health services and hospitals failed to identify any pneumonia case relevant to the spa center.

Environmental Investigation

This patient was found to have visited some local factories, a spa center, and his home in the 2 weeks preceding illness onset. Therefore, a total of 49 water samples from places, as described in Table 1, were collected and subjected to the isolation and enumeration of LP concentration (cfu/ml). However, only basin samples at the spa center were found to contain LP, with concentrations ranging from 3.2 to 44.5 cfu/ml in 10 hot basins and from 4.6 to 17.5 cfu/ml in 3 cold basins, respectively (Table 1). The basin-contamination rate at this spa center was thus calculated as 81.2% (13/16). In all, 13 LP strains, each represented an individual basin, were selected for further molecular subtyping.

Serogroup and Genomic Investigation

Two serogroups (i.e., SG 2 and SG 6) were identified among the 13 LP strains (Table 1). Genomic investigation of these 13 isolates further classified them into 5 PFGE-types (Fig. 1). Based on the serogroup and PFGE-typing, a total of 4 strains, including 3 strains (EN18-10, 11, and 17) from hot basin and 1 strain (EN19-4) from cold basin, were categorized as SG 2 and P2, which was identical to the pattern of the patient strain (CL-1). This strongly suggested the source of LG infection to be the spa basin.

Detection of Virulence Genes lvh *and* rtx*A in Isolates*

The virulence genes *lvh* and *rtx*A have a strong association with LG (26). Virulence PCR assay for both genes were, therefore, applied to LP isolates to determine if they played a role in this isolated case. As shown in Table 2, all 5 PFGE-types isolated in this study were identified as less- or non-virulent ones, lacking one (lvh^+, $rtxA^-$) or even two (lvh^-, $rtxA^-$) of the virulence genes (15). Noteworthy was that the LG-causative type in this infection (i.e., type P2, Table 2) was the only one to be present with *lvh* gene (lvh^+), indicating its relatively more virulent potential than those of other 4 PFGE-types.

Discussion

LP is ubiquitous in aquatic environments and commonly found in natural or man-made water sources (29). The two main sources of LP leading to infections in humans are cooling towers and potable water distribution systems (14). Taiwan's potable water distribution

Table 1. Serogroups and PFGE-typing of *Legionella pneumophila* strains isolated from this patient and his relevant environments

Sources of sample	Sample sum	*Legionella*		Isolates		Serogroup	PFGE type
		Positive sample	cfu/ml	Sum	Strain code[a]		
Water							
Factory faucet	21	0	0	—[b]	—	—	—
Home faucet	12	0	0	—	—	—	—
Spa center							
Hot basin	13	10	3.2–44.5[d]	10	EN18-1,2	SG6	P1
					EN18-10, 11, 17	SG2	P2[c]
					EN18-6, 7	SG2	P3
					EN18-3	SG2	P4
					EN18-4	SG2	P4a
					EN18-8	SG2	P5
Cold basin	3	3	4.6–17.5[d]	3	EN19-4	SG2	P2[c]
					EN19-3	SG2	P3
					EN19-2	SG2	P3a
Sputum							
Patient	1	1	na[e]	1	CL-1	SG2	P2[c]
Total	50	14			1 CL,13 EN	SG2, 6	P1–P5

[a] The clinical strain isolated from the patient's sputum was designated as CL-1, while those environmental strains from hot and cold basins were designated as EN-18 and EN-19, respectively.

[b] No bacteria strain available for analysis.

[c] Environmental strains categorized in this group have identical serogroup and PFGE-type with those of the CL-1 strain.

[d] The average cfu/ml of legionella in duplicate results of the ten positive hot-basin water samples were 3.2, 4.6, 6.5, 8.2, 9.7, 10.2, 11.6, 25.5, 31.8, and 44.5, while those of the three cold-basin samples were 4.6, 4.7, and 17.5, respectively.

[e] Not analyzed.

system, which is also the major water supplier of most city-spa centers, was first confirmed to contain LP in 1996 (24). Since then, only one case of LG has been reported to have its LP source originating from the residential water faucet (9). To our knowledge, spa-associated LG has never been described in Taiwan; neither has any outbreak of LG been reported here. It was the attempt of discovering any possible outbreak that prompted us to actively search for the source and additional cases besides this patient.

Our focus on the investigation of environmental water contamination, following the clues obtained from the interview of the patient's lifestyle features, proved to be correct in identifying the source of infection. The pattern of PFGE-type and serogroup strongly suggested the whirlpool water to be the source. However, no additional LG case was found, despite discovery in the whirlpool water of polymorphous LP strains.

This, therefore, raised two interesting questions regarding this natural infection in which at least 100 people were exposed to 5 genetic types of LP in a spa center. First, with more than 100 people registered as regular club members and, also, another about 100 irregular attendees per week, why was this patient the only one to develop LG from this public spa facility? Second, why was the P2 type of LP (Table 2), also, the only one out of the 5 PFGE-types, capable of causing infection in this incident?

To explore the first question, characteristics (or risk factors) unique to our patient, making him vulnerable to the infection of LP, were worth being sought out. To our knowledge, widely reported risk factors for LG included male sex, increasing age, smoking, heavy alcohol intake, and chronic illness (e.g., diabetes mellitus, chronic lung disease, renal disease, malignancy, immunocompromised status) (10, 14, 19, 28). Coincidently, data from our male patient literally recorded heavy smoking and alcoholism as his long-term habits, matching a total of 3 of the LG risk factors mentioned above. To the smoking factor in particular, medical evidence has already illustrated its contribution to the increased microbial infections by mechanisms of structural changes in the respiratory tract and a decrease in immune response (1). A 2- to 4-fold increased risk of invasive pneumococcal infection, incurred by cigarette smoking, has even been reported (1). Therefore, despite the lack of exact physical data to confirm the patient's deficiency in defending bacteria invasion, we are still inclined to regard him as a highly LG-susceptible host, based on the epidemiological risk-factors analysis.

As to the second question, virulent gene analysis was

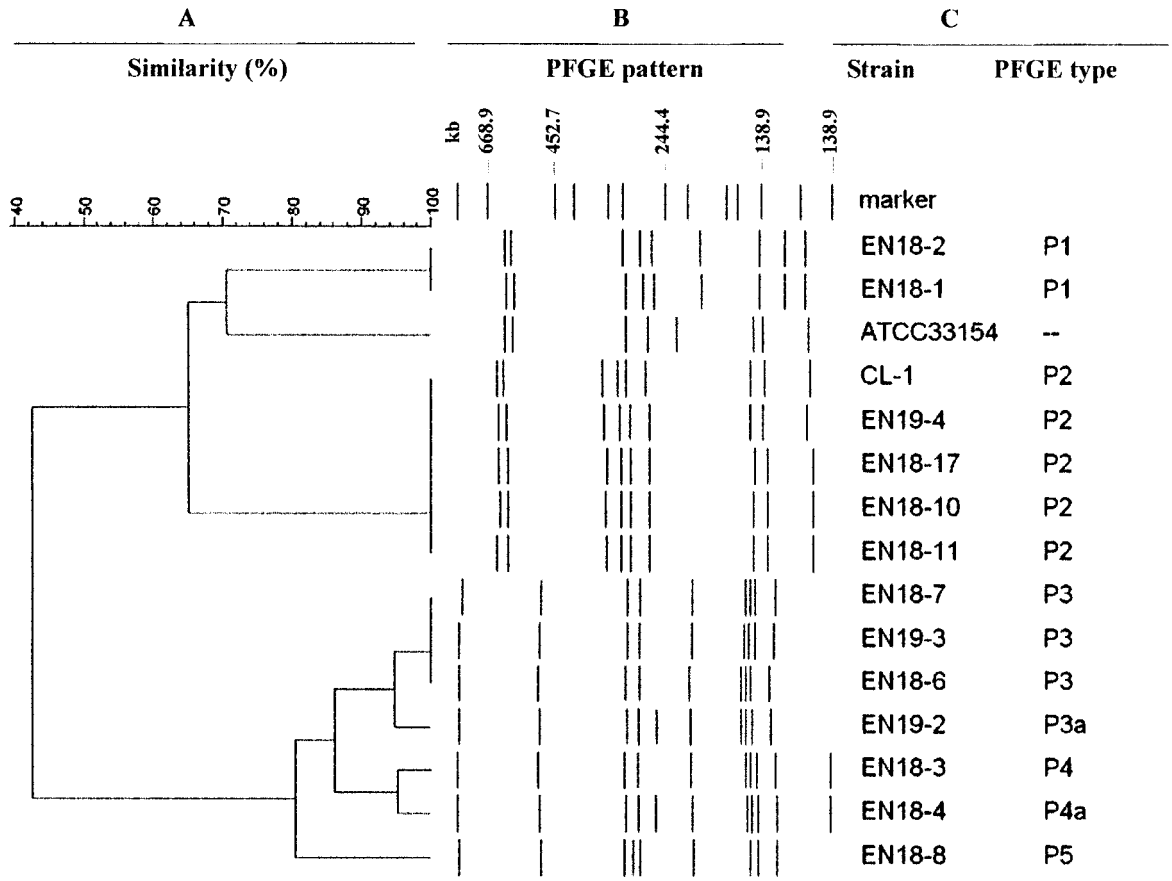

Fig. 1. (A) Dendrogram showing the relationship between strains of *Legionella pneumophila* (LP) based on PFGE data obtained with *Sfi*I as the restriction enzyme. Comparisons between the electrophoretic types obtained were made by using the Dice similarity coefficient and clustered by using the UPGMA algorithm (for more details, see the text). (B) PFGE patterns. Bacteriophage lambda concatamers (48.5 kb; New England Biolabs) was used as molecular weight standard. (C) Strain codes and PFGE-types of LP were identical to those in Table 1.

able to clearly classify our isolates into 2 groups, including the non-virulent (*lvh*$^-$, *rtx*A$^-$) and less-virulent (*lvh*$^+$, *rtx*A$^-$) types (Table 2). Noteworthily, the strong virulent type (*lvh*$^+$, *rtx*A$^+$), as usually seen in clinical strains elsewhere (15, 26) was not found in our spa samples. The majority (4 out of 5 PFGE-types) of our isolates belonged to the non-virulent type, which, as described in Huang (15), is incapable of causing disease in humans. Therefore, although the spa center contained polymorphous LP strains, only one of them, the less-virulent (*lvh*$^+$, *rtx*A$^-$) P2 type, had the potential of infecting people. Moreover, this type of less-virulent strain was suggested to possibly cause infection only when the immunity status and other risk factors of the patient are appropriate (15).

Taken together, the virulent potential of all LP strains in this spa center was not strong enough to provoke an outbreak, if spa attendants were all in good health. It was this patient, suggested to be weak in defending microbial attack, that the less-virulent LP strain got a chance to accomplish a successful infection. This mutual interaction between LP's virulent potential and patient's health-status was thus suggested to be the mechanism leading to an opportunistic infection in this isolated case.

Our first insight, through the virulent gene analysis, into the opportunistic mechanism may also provide an alternative explanation for the variation of reported attack rates in LG (i.e., 0.1–5%) (4, 10). Given the dynamic and complicated conditions naturally existing in the host's health-status and the degree of LP's virulent potential in whirlpool spas around the world, the results of their interactions, that is the attack rate, would certainly be very versatile.

Besides, to the best of our knowledge, serogroups of LP reported from spa-associated cases of LG thus far

Table 2. Presence of *lvh* and *rtx*A genes in *Legionella pneumophila* strains isolated in this study

PFGE type	Sero-type	Strains	Disease[a)]	*lvh*[b)]	*rtx*A[b)]	Source
P1	SG 6	EN18-1	−	−	−	This study
P2	SG 2	EN18-11	−	+	−	This study
P3a	SG 2	EN19-2	−	−	−	This study
P4	SG 2	EN18-3	−	−	−	This study
P5	SG 2	EN18-12	−	−	−	This study
P2	SG 2	CL-1	+	+	−	This study
na[c)]	SG 1	ATCC43109	+	+	+	ATCC
na	SG 1	ATCC33152	+	+	+	ATCC

[a)] Associated (+) or not (−) with disease in humans.
[b)] Both *lvh* and *rtx* genes were detected by PCR-amplification technique employing primers as described in Samrakandi et al.[26)]
[c)] Not analyzed.

included SG 1, 3, 4, 5, and 6 (2, 17, 20, 21, 23, 31). Accordingly, our opportunistic infection, by a low-virulent LP strain, has already added a new member, namely SG 2, to the record of SG involved in spa infection.

Since there is a small number of SG 2 in both clinical and environmental LP isolates around the world, we therefore scrutinized relevant papers trying to understand the distribution of SG 2 in Taiwan's pneumonia patients, potable water, and spa facilities. However, formal reports, which had their LP isolates determined to the serogroup level, were found to be very scanty here. Only three sporadic pneumonia cases were able to describe their LP pathogens as SG 1, SG 5, and SG 6, respectively (6, 7, 9). Larger scale investigation of pneumonia patients was seen only in Su (30), in which a total of 13 *Legionella* strains were isolated from the sputa of 237 patients and were identified as SG 1 (8/13, 61.5%), SG 3 (1/13, 7.7%), SG 6 (1/13, 7.7%), and SG 7 (1/13, 7.7%) of LP, and *L. dumoffi* (2/13, 15.3%), respectively. As for the potable water, a 20.2% (212/1,052) *Legionella*-positive rate of cooling towers was detected in a survey involving 1,052 samples collected around Taiwan in 1998 (8). In this survey, 59% (125/212) of the 212 LP isolates was identified as SG 1, while the remaining 41% (87/212) was classified as "Group SG 2–14" as a whole, because the commercial kit used was unable to specifically detect individual serogroups among SG 2–14 respectively. Failure to find clues of SG 2 elsewhere in Taiwan was also common to the aspect of spa water, because the only one documented report obtainable (16), in which a 14.2% (2/14) *Legionella*-positive rate of hot spring resorts was proclaimed, was found to have its 2 LP isolates eventually identified as SG 3 and SG 5, respectively. Therefore, our SG 2 culture turned out to be the first isolation in both clinical and environmental isolates on this island. Accordingly, more field surveys are still required to understand the real picture of SG 2 of LP in Taiwan.

Finally, although the spa center was seriously contaminated with LP, the water samples from faucets around the patient, which shared the same portable water supply system with the spa center, were negative in LP isolation. Obviously, the spa center was the site responsible for its own accumulation of polymorphous LP strains. Therefore, a suspension warrant was issued and the spa center was asked to drain all basins, change filters, and conduct a thorough cleansing and decontamination. As a negative result in culturing LP from the refilled whirlpool water was obtained, the spa center was allowed to reopen on May 20, 2005.

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