Fluorescent humic substances–arsenic complex in well water in areas where blackfoot disease is endemic in Taiwan

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The relationship between the four components, (1) fluorescence intensity, (2) arsenic concentration, (3) pH and (4) total dissolved solids, (TDS) measured in well waters from areas in Taiwan where blackfoot disease (BFD) is endemic was studied, as well as the relationships between the four degrees of BFD and each of the above four symptomatic components, in order to evaluate the etiological factors of BFD more progressively. The following 95% confidence intervals were obtained in well water samples ($n=1189$): fluorescence intensity, 26.837–32.570; arsenic concentration, 0.103–0.127 mg dm^{-3}; pH, 7.466–7.519; and TDS 733.063–801.647 mg dm^{-3}. Fluorescence intensities of the four degrees of BFD were not all the same ($F=64.54$, $P<0.001$), and nor were arsenic concentrations ($F=72.03$, $P<0.001$), pH values ($F=7.30$, $P<0.001$), nor TDS values ($F=10.76$, $P<0.001$). In addition, multiple comparisons indicate that the higher the epidemical degree, the higher the fluorescence intensities, arsenic concentrations and pH values become; however, such a relationship is not found for TDS values. Moreover, the fluorescence intensities have positive linear correlations with arsenic concentrations ($r=0.49$, $P<0.001$), pH ($r=0.25$, $P<0.001$), and TDS ($r=0.18$, $P<0.001$), as do the arsenic concentrations with pH ($r=0.22$, $P<0.001$). Of the four epidemical degree groups, pairs are not significantly different from one another in correlation coefficients between fluorescence intensity and arsenic concentration, which implies a steady relationship between fluorescent compounds and arsenic. We conclude that fluorescent compounds in well water, as possible etiological factors of BFD, are closely related to arsenic along with pH and TDS values in the areas where BFD is endemic. In addition, we infer that a complex is formed by fluorescent compounds, arsenic and other metals.

Keywords: Blackfoot disease, fluorescence intensity, arsenic, pH, TDS

INTRODUCTION

Blackfoot disease (BFD) is an endemic disease in the south-west coastal areas of Taiwan.[1] The first case reported was eight decades ago. The disease resembles so-called arteriosclerosis obliterans or thromboangitis obliterans in its consequences of ulceration and gangrene caused by the clinical features of numbness and sensation of coldness in distal extremities, rest pain, intermittent claudication, etc., and by the pathological evidence of peripheral vessel obliteration.[2]

To date, three arguments have been put forward on the pathogenesis of BFD: one is the general character of the well water in endemic areas, made by the earlier epidemiological investigations;[3] another is the presence of arsenic in the well water of the endemic areas, accredited by the statistics of public health researchers;[3] the third, put forward by us, is the presence of fluorescent compounds in the well water of the endemic areas. We have been engaged in fluorescent compound-related research since we detected fluorescent compounds in the well water of the BFD-endemnic areas.[4] Fluorescent compounds in the well water do play an important role in BFD pathogenesis factors.[5,6] Since pathogenic etiologies are controversial, we assume that the etiology of BFD is composed of many factors, not just one. For our experiments we drew well water in BFD areas. Through chemical analysis of the well water, its components were compared and contrasted. The purpose of the study was to investi-

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gate the relationships between well water components, viz. (1) relative fluorescence intensity, (2) arsenic concentration, (3) pH value, and (4) total dissolved solid (TDS), so as to find new causes for BFD etiology.

MATERIALS AND METHODS

With reference to an epidemiological survey of BFD,[7] we sampled water from 1189 wells in BFD-endemic areas in Taiwan (Yunlin county, Chiayi county, Tainan county, Kaohsiung county and Pingtung county). The 1189 wells were scattered among 261 villages in 40 towns of five counties: 211 wells from seven towns (including 47 villages) in Yunlin county; 378 wells from 12 towns (including 69 villages) in Chiayi county; 481 wells from 15 towns (including 113 villages) in Tainan county; 19 wells from one town (including eight villages) in Kaohsiung county and 100 wells from three towns (including 24 villages) in Pingtung county. The distributions of well samples from the five counties were: 17.7% (Yunlin county), 31.8% (Chiayi county), 40.5% (Tainan county), 1.6% (Kaohsiung county), and 8.4% (Pingtung county) (Fig. 1).

According to the Standard Degrees of Epidemicity of Blackfoot Disease' in south-west coastal areas, the well water was classified into the following four groups:[8]

- Degree 3 Endemic districts with BFD patients possessing skin symptoms
- Degree 2 Endemic districts where schoolchildren suffered from the skin symptoms of chronic arsenic intoxication but no BFD patients were found, or where BFD patients were found but no patient had skin symptoms
- Degree 1 Endemic districts where arsenic concentration in well water exceeded 0.35 mg dm^{-3} but no schoolchildren suffered from skin symptoms of chronic arsenic intoxication, and no BFD patients were found
- Degree 0 Endemic areas without the above features

The following methodology was used for the measurement of the four components of well water.

A Hitachi Model 204 automatic recording fluorescence spectrophotometer was used to measure relative fluorescence intensity, with exciting wavelength 340 nm, emission wavelength 415 nm, sensitivity control 4, selector ×10, and the standard reagent, quinine sulphate (5×10^{-5} mg cm^{-3}), at 21 relative fluorescence intensity units.

Using a Perkin–Elmer Model 2380 atomic absorption spectrophotometer, the mercury hydride system was used to measure the arsenic concentration (units: mg dm^{-3}). The sample water, collected in a bottle, was transferred to another bottle; 10 cm^3 of concentrated hydrochloric acid was added to the empty bottle, which was stoppered and the inside of the bottle was rinsed with the acid to dissolve any material adhering to the wall. The bottle was left to stand for 1 h, and the sample water was then returned to the bottle. Then the sample solution was analysed. The wavelength was 194 nm, lamp 12.2 w, and slit width 0.7 nm (mg/L). These procedures were adopted from those of Natelson.[9]

The pH value was measured at 25 °C with a Fisher Model 230 pH-meter. A conductivity meter Model 5003 (Sprite Industries, USA) was used for direct measurement of TDS values (unit: mg dm^{-3}).

All calculations were completed by IBM PC/XT with LOTUS 1-2-3 Release 2.01. Biostatistical methods were as follows: (1) one-way analysis of variance (one-way ANOVA); (2) Student–Newman–Keuls (SNK) multiple comparison test; (3) simple linear regression and correlation; and (4) Fisher's z test.

RESULTS AND DISCUSSION

Under the same conditions, relative fluorescence intensity, arsenic concentration, pH value and TDS value were measured at 1189 wells, the sampled water of which was classified into the different degrees of epidemicity. Wells with Degree 3 amounted to 195 (16.4%); wells of Degree 2 to 157 (13.2%); wells of Degree 1 to 458 (38.5%); wells of Degree 0 to 379 (31.9%) (fig. 1).

Listing the means (±SE) and 95% confidence intervals of fluorescence intensity, arsenic concentration, pH value, and TDS value in well water from different degrees of epidemicity (Table 1) indicates that the higher the epidemical

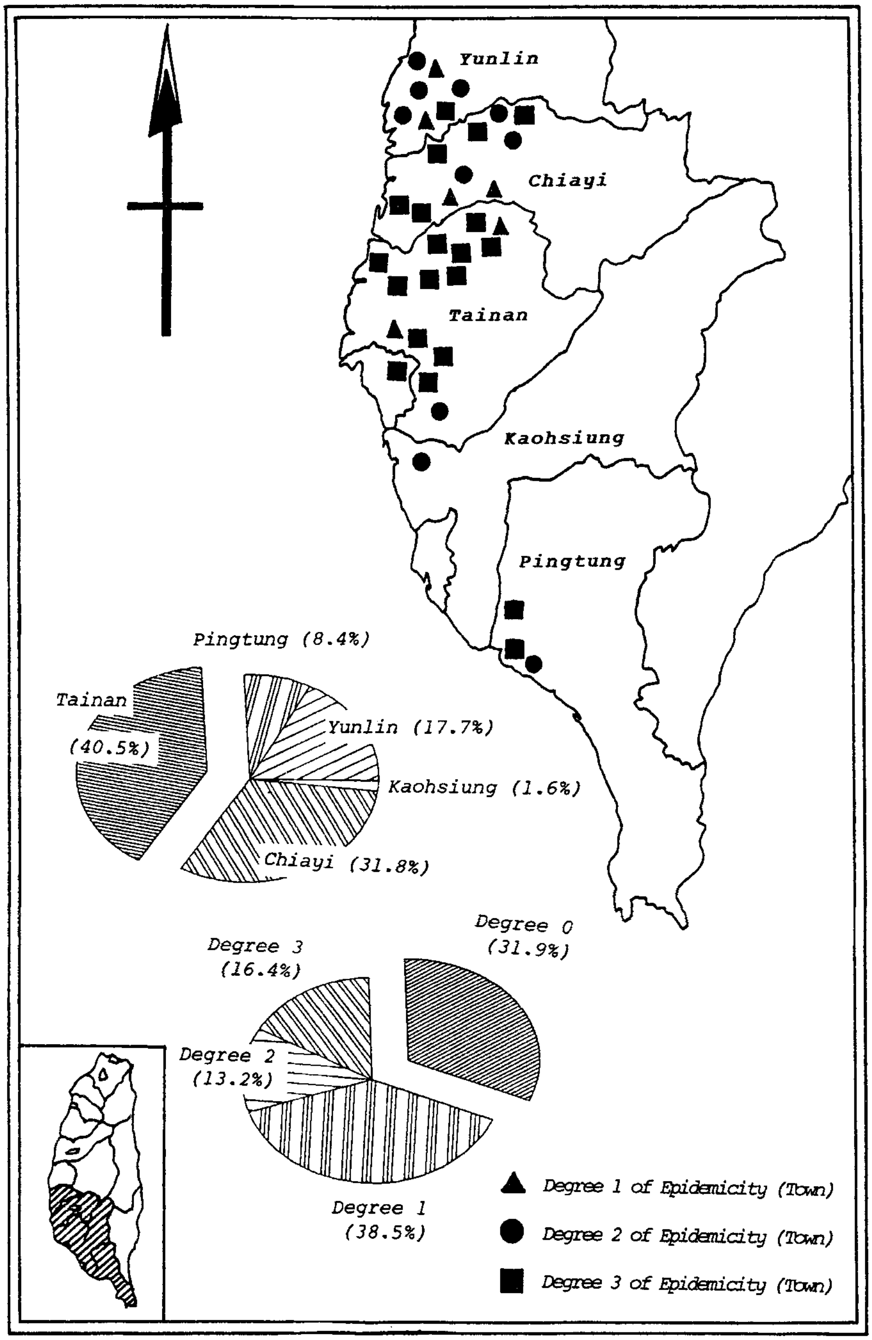

Figure 1 Maps of localities (towns) in Taiwan where the wells were sampled from the BFD endemic areas, with pie charts of distribution ratios of the well samples from areas of the four epidemical degrees and from the BFD-endemic counties.

Table 1 Mean fluorescence intensity, arsenic concentration, pH and TDS values for different epidemical degrees of BFD[a]

		Mean ± SE (95% CI)[a]			
Degree of epidemicity	No. of subjects	Fluorescence intensity	As conc[b] (mg dm^{-3})	pH	TDS (mg dm^{-3})
0	379	16.69 ± 1.13 (14.46, 18.91)	0.05 ± 0.01 (0.04, 0.06)	7.45 ± 0.03 (7.40, 7.51)	778.69 ± 32.70 (714.39, 843.00)
1	458	23.38 ± 1.28 (20.88, 25.89)	0.08 ± 0.01 (0.07, 0.09)	7.46 ± 0.02 (7.42, 7.50)	666.50 ± 19.48 (628.20, 704.79)
2	157	27.68 ± 2.13 (23.48, 31.88)	0.19 ± 0.02 (0.15, 0.23)	7.51 ± 0.04 (7.44, 7.58)	807.80 ± 52.42 (704.25, 911.35)
3	195	71.48 ± 7.19 (57.29, 85.66)	0.27 ± 0.02 (0.22, 0.31)	7.63 ± 0.03 (7.56, 7.69)	949.64 ± 56.34 (838.53, 1060.76)
Total	1189	29.70 ± 1.46 (26.84, 32.57)	0.11 ± 0.01 (0.10, 0.13)	7.49 ± 0.01 (7.47, 7.52)	767.35 ± 17.48 (733.06, 801.65)

[a]Values are given as mean ± SE (standard error of the mean) with 95% CI (confidence interval) in parentheses. TDS, total dissolved solids. [b]As concn., arsenic concentration.

degree, the higher the sample means of fluorescence intensity, arsenic concentration and pH value become, but not that for TDS value.

Relationships between epidemical degree and fluorescence intensity, arsenic concentration, pH and TDS value in well water

The results of one-way analysis of variance (one-way ANOVA) are shown in Table 2. We conclude that at least two of the means of fluorescence intensity ($F = 64.54$, $d_1 = 3$, $d_2 = 1185$, $P < 0.001$ where d is degree of freedom), arsenic concentration ($F = 72.03$, $d_1 = 3$, $d_2 = 1185$, $P < 0.001$), TDS value ($F = 10.76$, $d_1 = 3$, $d_2 = 1185$, $P < 0.001$), and pH ($F = 7.30$, $d_1 = 3$, $d_2 = 1185$, $P < 0.001$) are not the same between epidemical degrees (Table 2).

The results of Student–Newman–Keuls (SNK) multiple comparison procedures for all possible pairs of groups of different epidemical degrees, in fluorescence intensity, arsenic concentration, pH value and TDS value, are displayed in Table 3. With regard to fluorescence intensity, Degree 1 areas are commensurate with Degree 2 areas. These two degrees are significantly higher than for Degree 0 areas and lower than for Degree 3 areas. With respect to arsenic concentration, Degree 0 areas are significantly lower than Degree 1 areas; Degree 1 areas are lower than Degree 2 areas; Degree 2 areas are lower than Degree 3 areas. With regard to TDS value, Degree 0 areas are commensurate to Degree 2

Table 2 Comparisons of fluorescence intensity, arsenic concentration, pH and TDS value in the four epidemical degrees of BFD

	Epidemical degree of blackfoot disease									
	0 ($n = 379$)		1 ($n = 458$)		2 ($n = 157$)		3 ($n = 195$)			
	Mean	SD[a]	Mean	SD	Mean	SD	Mean	SD	F statistic	P
Fluorescence intensity	16.69	22.01	23.38	23.30	27.68	26.65	71.48	100.44	64.54	<0.001
As conc. (mg dm^{-3})	0.05	0.10	0.08	0.11	0.19	0.27	0.27	0.33	72.03	<0.001
pH	7.45	0.52	7.46	0.40	7.51	0.45	7.63	0.47	7.30	<0.001
TDS (mg dm^{-3})	778.69	636.68	666.50	417.00	807.80	656.81	949.64	786.68	10.76	<0.001

[a]SD, standard deviation of the mean.

Table 3 Multiple comparisons of fluorescence intensity, arsenic concentration, pH and TDS values in the four epidemical degrees of BFD by the Student–Newman–Keuls (SNK) test

	Studentized range statistic q value $\langle C\rangle$[b]			
Range[a]	Fluorescence intensity	As concn (mg dm^{-3})	pH	TDS (mg dm^{-3})
D(3) to D(0) [(D(3) to D(1)][a]	18.80 ⟨4⟩**	18.56 ⟨4⟩**	6.09 ⟨4⟩**	7.87 ⟨4⟩**
D(2) to [D(2) to D(1)]	3.50 ⟨3⟩*	10.90 ⟨3⟩**	1.92 ⟨3⟩	3.63 ⟨3⟩*
D(3) to D(1) [D(3) to D(0)]	17.01 ⟨3⟩**	16.80 ⟨3⟩**	5.96 ⟨3⟩**	4.61 ⟨3⟩**
D(1) to D(0) [D(0) to D(1)]	2.2 ⟨2⟩*	2.87 ⟨2⟩*	0.39 ⟨2⟩	3.84 ⟨2⟩**
D(2) to D(1) [D(2) to D(0)]	1.40 ⟨2⟩	9.03 ⟨2⟩**	1.68 ⟨2⟩	0.73 ⟨2⟩
D(3) to D(2) [D(3) to D(2)]	12.35 ⟨2⟩**	5.60 ⟨2⟩**	3.30 ⟨2⟩*	3.14 ⟨2⟩*

[a]D(0), Degree 0 of epidemicity; D(1), Degree 1 of epidemicity; D(2), Degree 2 of epidemicity; D(3), Degree 3 of epidemicity. [b]C, number of means in the range. [c][ ], range for TDS. **$P<0.01$. *$P<0.05$.

areas. These two Degrees are significantly higher than for Degree 1 areas and lower than for Degree 3 areas. With respect to pH value, Degree 0, Degree 1 and Degree 2 areas are commensurate with one another, and significantly lower than for Degree 3 areas.

Relationships between fluorescence intensity, arsenic concentration, pH and TDS value in well water

A positive linear correlation between fluorescence intensity and arsenic concentration ($r=0.49$, d.f. $=1187$, $P<0.001$) in sampled well water suggests that the higher the fluorescence intensity, the higher the arsenic concentration becomes.

A positive linear correlation between fluorescence intensity and TDS value ($r=0.18$, d.f. $=1187$, $P<0.001$) in sampled well water indicates that the higher the fluorescence intensity, the higher the TDS value becomes.

A positive linear correlation between fluorescence intensity and pH value ($r=0.25$, d.f. $=1187$, $P<0.001$) in sampled well water demonstrates that the higher the fluorescence intensity, the higher the pH value becomes.

A positive linear correlation between arsenic concentration and pH value ($r=0.22$, d.f. $=1187$, $P<0.001$) in sampled well water suggests that the higher the arsenic concentration, the higher the pH value becomes.

Comparisons of correlation coefficients between fluorescence intensity and arsenic concentration

Table 4, showing an insignificant difference between each pair of correlation coefficients between the fluorescence intensity and arsenic concentration of the different epidemicity groups by Fisher's z-test, reveals the stable relationship between fluorescent compounds and arsenic concentration.

CONCLUSIONS

The areas investigated covered all the BFD endemic districts, from Yunlin county (northernmost) to Pingtung county (southernmost) and the 1189 wells sampled included all the four epidemical degree areas.

As the results show, arsenic concentrations (0.11 ± 0.20 mg dm^{-3}, $n=1189$) (Table 1) in well waters were not as high as we had expected. Even in Degree 3 areas, arsenic concentrations in well waters were also lower than the expected value of 0.35 mg dm^{-3}. Possible reasons for the previous overestimate of arsenic concentrations were as follows: (1) the earlier analytical instruments and methods have been improved; (2) diversity of geographical environment; and (3) different areas and different depths of wells from before were investigated this time.

Table 4 Fisher's *z* test for comparing two correlation coefficients between the fluorescence intensity and arsenic concentration in pairs of the four epidemical degrees of BFD[a]

Correlation coefficients	D(0) ($n=379$)	D(1) ($n=458$)	D(2) ($n=157$)	D(3) ($n=195$)
r	0.43	0.42	0.38	0.46

D(0) vs D(1) $P>0.05$	D(1) vs D(2) $P>0.05$
D(0) vs D(2) $P>0.05$	D(1) vs D(3) $P>0.05$
D(0) vs D(3) $P>0.05$	D(2) vs D(3) $P>0.05$

[a]D(0): Degree 0 of epidemicity; D(1): Degree 1 of epidemicity; D(2): Degree 2 of epidemicity; D(3): Degree 3 of epidemicity.

In the past, only arsenic concentrations in well waters were considered as having direct correlation with the epidemical degrees of BFD, but the results of this investigation indicate that fluorescence intensity also has direct correlation. Moreover, in well water, the higher the fluorescence intensity, the higher the arsenic concentration, pH and TDS value become, and that implies not only arsenic but also pH value and TDS being closely related to fluorescent compounds.

Both fluorescent compounds and arsenic in well waters therefore have a direct correlation with the epidemical degree of BFD, fluorescent compounds are a form of humic substance,[5] i.e. a polymer with a multiplicity of anions, having strong chelating ability which may therefore bind many positively charged metal cations and other compounds. In the process of chelation, arsenic in well water can combine with fluorescent compounds to become a complex. The evidence of a stable relationship between fluorescent compounds and arsenic supports the above inference. Because of the existence of complexes between fluorescent compounds and arsenic, both arsenic and fluorescent compounds reveal a direct relationship with the epidemical degrees of BFD. It remains to be considered if arsenic alone or fluorescent compounds (humic substances) alone, is the causal factor having direct relationship with the epidemical degree of BFD.

The authors had noticed before that in endemic areas of BFD, the pH values of well water had special characteristics, and had stated that pH value and TDS value in well water had some relationship with the epidemical degree of BFD.[4] Whether these two factors may have some effect on the combination of fluorescent compounds with arsenic still needs more investigation.

In endemic areas of BFD, well water does not meet the standard criteria of drinking water with respect to TDS values (733.098–801.612 ppm) (Table 1).

To conclude this research has shown that not only arsenic but also fluorescent compounds in well water have a direct relationship with the epidemical degree of BFD. Since arsenic and fluorescent humic substances in well water are probably combined in complexes, we cannot ascertain which, viz: arsenic or fluorescent humic substances, in well water, has a direct and causal relationship with the epidemical degree of BFD.

Tables of data (arsenic concentrations, TDS, fluorescent intensity, pH) and detailed statistical results are lodged with the editor.

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