郵送調査とは逆の方向に補正が働いているように見える。

そこで、傾向スコアウエイトによる推定量補正状況を、より詳細に飼育犬頭数の分布から見ることとする。図 1 は、SPBI 世帯人員ウエイト, SPBP 世帯人員ウエイト, SPBI 傾向スコアウエイトによる飼育犬頭数分布を示したものである。黒と赤は世帯人員ウエイトによる SPBI と SPBP を示したものであり、頭数 0 がインターネット調査で多いのに対し、頭数 1 以上では郵送調査が上回っていることがわかる。青が SPBI の傾向スコアウエイトによる補正結果であり、完全ではないものの郵送調査の分布に近づいていることがわかる。ただし、飼育頭数が 0 および 1 に関しては補正がかなり有効に働いているのに対し、飼育頭数が多い 2 及び 3 以上に関してはあまり補正が効いていないことが観察され、これが「1 世帯あたり平均飼育頭数(犬を飼育している世帯)」の指標の逆向きの動きに関連していると考えられる。しかしながら、全体の分布の形状としては、かなり郵送調査の分布に近づく補正が行われていると考えられよう。

これを JPFA に適用したものが図 2 である。これを見ると、図 2 と同様の形で飼育犬頭数分布が補正されていることがわかる。このように、傾向スコアウエイト調整によって、インターネット調査が持つ有意抽出に起因するバイアスを一定程度補正することが可能である。

なお、SPBP はサンプルサイズがあまり大きくない(全体で 1,140 サンプル、20〜69 歳は 966 サンプル)ことから、この補正の有効性については検証が必要であると考えられる。JGSS では飼育犬頭数は調査されていないが、飼育の有無については調査が行われており、サンプルサイズも 2,130(ペット関する調査が行われている留置調査票 B 票の対象)、そのうち 20〜69 歳は 1,755 となっており、2 倍弱程度のサンプルサイズを持つため、これとの比較から SPBP の飼育世帯数割合が評価できると考えられる。そこで、JGSS において世帯人員ウエイト調整を行った飼育世帯数割合を算出すると、20.5%となり、これは、SPBP の 21.2%よりやや低い値となっている。両者は調査時点が違うことなどから単純に比較することはできないが、SPBP の水準はやや JGSS より高いものの、大きく異なる水準ではないといえよう。したがって、SPBP を用いた傾向スコアウエイトによる補正は、やや過大となる可能性はあるものの、概ね妥当なものであると考えることができるだろう。

(3)飼育犬頭数推計

(1)において、2010 年国勢調査ベースの世帯数について、20 歳未満のみの世帯員で構成される世帯、20〜69 歳が少なくとも一人以上いる世帯、70 歳以上のみで構成される世帯に分けて推計を行った。このように分けた背景として、B において、「全国犬・猫飼育実態調査」では対象者が 20〜69 歳の男女に限定されていて、20 歳未満のみ、あるいは 70 歳以上のみの世帯員で構成される世帯の実態は把握することができないことを挙げた。そして、20 歳未満のみの世帯員で構成される世帯の飼育犬頭数は無視できるレベルである一方、70 歳以上のみの世帯員で構成される世帯については、飼育犬は存在すると考えられるものの、飼育率や平均飼育頭数はそれ以外の世帯より低いレベルにあると考えられることを述べた。そこで、これらの要因を考慮し、以下のように総飼育犬頭数推計法を提案する。

(k)で世帯のグループ、すなわち、$k=1$: 20歳未満のみで構成される世帯、$k=2$: 20～69歳が少なくとも一人以上いる世帯、$k=3$:70 歳以上のみで構成される世帯を表すこととし、$\bar{X}^{(k)}$: グループ(k)の平均飼育頭数、$S^{(k)}$: グループ(k)の総世帯数とする。このとき、総飼育犬頭数$\tilde{T}$は、

$$\tilde{T}=\sum_k \tilde{\bar{X}}^{(k)}S^{(k)}=\sum_k R_b{}^{(k)}\tilde{\bar{X}}_b^{(k)}S^{(k)}$$

で表される。ここで、$R_b{}^{(k)}$はグループ(k)で犬を飼育している世帯数割合、$\tilde{\bar{X}}_b^{(k)}$はグループ(k)の 1 世帯あたり平均飼育頭数(犬を飼育している世帯)である。

グループ(2)については、(2)において推計を行った、飼育世帯数割合及び 1 世帯あたり平均飼育頭数(犬を飼育している世帯)を用いればよく、また、グループ(1)については$R_b{}^{(1)}=0$と考えればよいので、問題になるのは全く情報がないグループ(3)となる。ここで、JGSS を用いると、70 歳以上のみの世帯員で構成される世帯についての飼育世帯数割合については算出が可能である。そこで、JGSS におけるグループ(k)の飼育世帯数割合を$R_b{}^{(k),JGSS}$と書くと、世帯人員ウエイト調整した値で、$R_b{}^{(2),JGSS}=0.20512$、$R_b{}^{(3),JGSS}=0.03562$となる。しかしながら、$\tilde{\bar{X}}_b^{(k)}$については JGSS からも情報がないことと、飼育世帯についての平均飼育頭数は大きく違わないと考えられることから、$\tilde{\bar{X}}_b^{(3)}=\tilde{\bar{X}}_b^{(2)}$と仮定し、以下の式により総飼育犬頭数を推計する。

$$\tilde{T}=R_b{}^{(2)}\tilde{\bar{X}}_b^{(2)}S^{(2)}+R_b{}^{(2)}\frac{R_b{}^{(3),JGSS}}{R_b{}^{(2),JGSS}}\tilde{\bar{X}}_b^{(2)}S^{(3)}$$

この推定法に基づき、総飼育犬頭数を推計したのが表 7 である。JFPA を用い、世帯グループを考慮し、傾向スコアウエイト調整を行った推計値は 1,004 万 4 千頭となる。

D. 考察

現在、「全国犬・猫飼育実態調査」では、総飼育犬頭数の推定値を 11861 千頭としている。本研究では、この推計において、「補助変量として用いられている世帯総数」推計及び「1 世帯あたりの平均飼育頭数」推計の 2 つの観点から推計方法及び推計値に関して評価を行った。

「補助変量として用いられている世帯総数」推計については、表 7 によれば、調整なし(人口ウエイトのみ)の JPFA による総飼育犬頭数推計値は 10067 千頭となり、オリジナルの「全国犬・猫飼育実態調査」結果 11861 千頭より 15.1%低いものとなった。これは、補助変量として施設なども含む住民基本台帳による世帯総数を用いていること、また、20 歳未満や 70 歳以上のみで構成される世帯などを同様に取り扱っていることに起因するバイアスと評価できる。

一方、「1 世帯あたりの平均飼育頭数」推計の影響については、さらに、個人データを用いた推計法に関する点とインターネット調査が有意抽出であることに起因する点の 2 つに分けて評価を行った。表 7 によれば、世帯人員ウエイトの JFPA による推計値は 8171 千頭であり、調整なし(人口ウエイトのみ)より 18.8%低く、これが個人データを用いた推計法に関するバイアスとなる。一方、傾向スコアウエイトによる推計値は 10044 千頭となっ

ており、世帯人員ウエイトによる推計値より 22.9% 高いものとなっている。これがインターネット調査が有意抽出であることに起因するバイアスとなる。したがって、両者を合わせると、1 世帯あたりの平均飼育頭数推計法が総飼育犬頭数の推定値に与えている影響は 0.2%((1−0.188)×1.229=0.998) 程度であると評価できる。すなわち、「全国犬・猫飼育実態調査」における1世帯あたりの平均飼育頭数は上方・下方両者のバイアスが相殺し、結果としては偏りの少ない推定値となっていると考えられる。

本研究で行った評価から、「全国犬・猫飼育実態調査」を用いた総飼育頭数の推計にあたって、以下が提言できる。

・「補助変量として用いられている世帯総数」については、住民基本台帳ベースではなく、国勢調査の一般世帯ベースにするとともに、沖縄県を含め、20 歳未満や 70 歳以上のみで構成される世帯についても考慮することが望ましい。

・「1 世帯あたりの平均飼育頭数」については、現在の値も偏りが少ないと考えられることから、補正を行う必要性は大きいものではないが、今後もバイアスの動向等に関する検証を続けていくことが必要である。

なお、今回提案した推計方法については、種々の不確実な要素が含まれていることや、改善可能と考えられる点があることから、これを確定的なものと捉えるのではなく、今後も検討を続けていく必要があると考える。

具体的には、本研究では、平成 22(2010)年の国勢調査ベース世帯数を種々の統計を用いて推計したが、平成 22(2010)年は国勢調査が実施された年であり、この調査結果が明らかになれば、より信頼性の高い補助変量として利用することが可能となる。また、世帯人員ウエイトについては、JGSS を用いて簡易的な補正を行ったが、JPFA で 20〜69 歳にかかる同居世帯人員数が調査可能であれば、直接ウエイトをかけて集計することが可能である。傾向スコアウエイトについては、本研究では現在の JPFA で利用可能な項目に限ってロジスティック回帰分析を行ったが、将来、より有効な調査項目を提案する観点から、SPB を用いて有効な変数を研究することも必要であろう。これらについては、今後の研究課題としたい。

最後に、本研究では比較的小規模な調査を用いて、インターネット調査の補正に関する検討を行ったが、小規模な調査から推定を行うことには限界もある。可能であれば、一回、大規模な無作為抽出標本を用いて飼育犬頭数に関する実態把握を行い、それとインターネット調査の関連を分析した上で、それ以降、これを利用してインターネット調査の補正を行っていくことが望ましい。その際には、本研究の研究成果も活かされることとなろう。政府統計において、今後、大規模な無作為抽出標本調査による飼育犬頭数実態把握の実施が望まれる。

E. 結論

本研究では、わが国における飼育犬頭数の推計精度を高めることを目的として、推定手法に関する検討を行った。「全国犬・猫飼育実態調査」では 1 世帯あたりの平均飼育頭数を推計し、さらにこれに世帯総数を乗じるという比推定が採られており、推定手法の検討にあたっては、「補助変量として用いられている世帯総数」と「1 世帯あたりの平均飼育頭数」に分けて評価を行うことが必要

である。

「補助変量として用いられている世帯総数」推計については、「全国犬・猫飼育実態調査」では補助変量として施設なども含む住民基本台帳による世帯総数を用いていること、また、20歳未満や70歳以上のみで構成される世帯などを同様に取り扱っていることからバイアスが発生しており、これを補正すると総飼育犬頭数は10067千頭となり、オリジナルの「全国犬・猫飼育実態調査」結果11861千頭より15.1%低いものとなった。

一方、「1世帯あたりの平均飼育頭数」推計の影響については、さらに、個人データを用いた推計法に関する点とインターネット調査が有意抽出であることに起因する点の2つに分けて評価を行った。個人データを用いた推計法に関するバイアスを補正すると総飼育犬頭数は18.8%低くなるのに対して、インターネット調査が有意抽出であることに起因するバイアスを補正すると推計値は22.9%高いものとなることから、1世帯あたりの平均飼育頭数推計法が総飼育犬頭数の推定値に与えている影響は0.2%((1-0.188)×1.229=0.998)程度であると評価された。すなわち、「全国犬・猫飼育実態調査」における1世帯あたりの平均飼育頭数は上方・下方両者のバイアスが相殺し、結果としては偏りの少ない推定値となっていると考えられる。これらを織り込んだ傾向スコアウエイトを用い、全国犬・猫飼育実態調査」による総飼育犬頭数を補正すると10044千頭となり、オリジナルの「全国犬・猫飼育実態調査」結果11861千頭より15.1%低いものとなった。

本研究で行った評価から、「全国犬・猫飼育実態調査」を用いた総飼育頭数の推計にあたって、以下が提言できる。

・「補助変量として用いられている世帯総数」については、住民基本台帳ベースではなく、国勢調査の一般世帯ベースにするとともに、沖縄県を含め、20歳未満や70歳以上のみで構成される世帯についても考慮することが望ましい。

・「1世帯あたりの平均飼育頭数」については、現在の値も偏りが少ないと考えられることから、補正を行う必要性は大きいものではないが、今後もバイアスの動向等に関する検証を続けていくことが必要である。

今回提案した推計方法については、種々の不確実な要素が含まれていることや、改善可能と考えられる点があることから、これを確定的なものと捉えるのではなく、今後も検討を続けていく必要があると考える。また、本研究の研究成果を活かす観点からも、政府統計において、今後、大規模な無作為抽出標本調査による飼育犬頭数実態把握の実施が望まれる。

なお、推計された頭数を元にすると、平成21年度に狂犬病予防注射を受けた犬は約半数に留まることになり、これを踏まえ、国内での狂犬病発生時の対応の検討や狂犬病対策に関する普及啓発を一層推進することが重要である。

F. 健康危険情報

G. 研究発表

1. 論文発表

なし

2. 学会発表

なし

H. 知的財産権の出願・登録状況

なし

1. 特許取得

なし

2. 実用新案登録

なし

3. その他

なし

表 1　世帯数の比較 (千世帯)

年次	住民基本台帳	国勢調査	国民生活基礎調査
2000	47,420	46,782	45,545
2001	48,015	-	45,664
2002	48,638	-	46,005
2003	49,261	-	45,800
2004	49,838	-	46,323
2005	50,382	49,063	47,043
2006	51,102	-	47,531
2007	51,713	-	48,023
2008	52,325	-	47,957
2009	52,878	-	48,013
2010	53,363	-	-

表 2　平均同居世帯人員数 (20〜69 歳) (人, JGSS)

	犬飼育なし		犬飼育あり	
年齢階級	男性	女性	男性	女性
20-24	2.77	2.86	3.31	3.29
25-29	2.80	2.78	3.27	3.30
30-34	2.42	2.20	3.21	3.20
35-39	2.07	2.21	2.69	2.33
40-44	1.98	2.05	2.08	2.41
45-49	2.08	2.38	2.71	2.58
50-54	2.36	2.84	2.86	2.91
55-59	2.67	2.56	3.00	2.83
60-64	2.46	2.44	2.76	2.50
65-69	2.41	1.99	2.50	2.29

表3　平均同居世帯人員数の比率（インターネット調査／郵送調査）

年齢階級	犬飼育なし		犬飼育あり	
	男性	女性	男性	女性
20-29	0.80	0.72	1.01	1.03
30-39	0.80	0.77	0.60	0.85
40-49	0.79	0.72	0.91	0.74
50-59	0.87	0.97	1.01	1.16
60-69	0.66	0.81	0.75	0.78

表4　ロジスティック回帰分析結果

	Estimate	Std. Error	t value	Pr(>|t|)
(Intercept)	0.6508	0.3273	1.99	0.0469
sex	-0.0836	0.1064	-0.79	0.4324
Q4_1_1	-0.8344	0.4370	-1.91	0.0563
Q4_1_2	0.1289	0.2640	0.49	0.6254
Q4_1_3	0.3385	0.4066	0.83	0.4052
Q4_1_4	-0.2758	0.2580	-1.07	0.2852
Q4_1_5	-0.2031	0.7240	-0.28	0.7791
Q4_1_6	-0.2770	0.3763	-0.74	0.4617
Q4_1_7	0.1735	1.3192	0.13	0.8954
Q4_1_8	0.1597	0.3160	0.51	0.6134
Q4_1_9	0.7416	0.3884	1.91	0.0564
Q4_1_10	1.1082	0.5516	2.01	0.0447
Q4_1_11	-2.2220	0.9564	-2.32	0.0202
Q4_1_12	-0.3066	0.5103	-0.57	0.5705
Q4_1_13	0.6930	1.2166	0.57	0.5690
Q4_1_14	0.1982	0.3738	0.53	0.5960
Q4_1_15	0.2825	0.2257	1.25	0.2108
Q4_2_1	-0.2997	0.2016	-1.49	0.1373
Q4_2_2	-0.0798	0.2055	-0.39	0.6977
Q4_2_3	0.2887	0.2141	1.35	0.1777
Q4_2_4	-0.1604	0.1694	-0.95	0.3440
Q4_2_5	0.1637	0.5069	0.32	0.7468
Q4_2_6	0.2296	0.2753	0.83	0.4043
Q4_2_7	0.6026	0.6730	0.90	0.3707
Q4_2_8	-0.3801	0.2433	-1.56	0.1185
Q4_2_9	-0.6597	0.2887	-2.29	0.0224
Q4_2_10	-0.7031	0.4053	-1.73	0.0829
Q4_2_11	0.9139	0.5898	1.55	0.1214
Q4_2_12	0.0242	0.3782	0.06	0.9490
Q4_2_13	0.0689	0.6356	0.11	0.9136
Q4_2_14	-0.0143	0.2194	-0.07	0.9479
Q4_2_15	0.3294	0.1822	1.81	0.0707
Q4_3_1	-0.4098	0.1751	-2.34	0.0193
Q4_3_2	0.1975	0.1845	1.07	0.2846
Q4_3_3	-0.3577	0.2929	-1.22	0.2221
Q4_3_4	0.6125	0.3027	2.02	0.0431
Q4_3_5	-1.4291	0.6731	-2.12	0.0338
Q4_3_6	-0.3137	0.2727	-1.15	0.2501
Q4_3_8	-0.0336	0.3303	-0.10	0.9190
Q4_3_9	0.6062	0.4798	1.26	0.2066
Q4_3_10	-0.2038	0.6166	-0.33	0.7410
Q4_3_11	0.7201	0.6194	1.16	0.2451
Q4_3_12	0.5476	0.3063	1.79	0.0740
Q4_3_13	1.0519	0.8309	1.27	0.2056
Q4_3_14	0.3207	0.3276	0.98	0.3276
Q4_3_15	-0.8449	0.1911	-4.42	0.0000
Q7_1	1.0542	0.3813	2.76	0.0057
Q7_2	0.1877	0.2946	0.64	0.5210
Q7_3	0.3349	0.3335	1.00	0.3154
Q7_4	0.0333	0.2561	0.13	0.8966
Q7_5	0.6598	0.3682	1.79	0.0732
Q7_6	-0.1131	0.3681	-0.31	0.7587
Q7_7	-0.4676	0.5873	-0.80	0.4260
Q9	0.0152	0.0906	0.17	0.8670
age2	0.0277	0.1685	0.16	0.8694
age3	-0.0505	0.1653	-0.31	0.7599
age4	0.0316	0.1674	0.19	0.8505
age5	0.0687	0.1627	0.42	0.6730
area2	-0.3653	0.3067	-1.19	0.2338
area3	-0.8292	0.3249	-2.55	0.0108
area4	-0.6315	0.3150	-2.00	0.0451
area5	-0.3812	0.2426	-1.57	0.1162
area6	0.0442	0.4256	0.10	0.9173
area7	-0.5202	0.2658	-1.96	0.0504
area8	-0.3891	0.2569	-1.51	0.1300
area9	-0.1190	0.3098	-0.38	0.7009
area10	0.0558	0.3863	0.14	0.8853
area11	-0.1080	0.2753	-0.39	0.6948

表5　犬を飼育している世帯数割合 (%)

	JFPA	SPBI	SPBP
調整なし (人口ウエイトのみ)	17.8	17.5	23.9
世帯人員ウエイト	14.5	14.5	21.2
傾向スコアウエイト	18.6	18.5	·

表6　1世帯あたり平均飼育頭数 (犬を飼育している世帯)

	JFPA	SPBI	SPBP
調整なし (人口ウエイトのみ)	1.26	1.20	1.32
世帯人員ウエイト	1.25	1.20	1.32
傾向スコアウエイト	1.21	1.17	·

表7　総飼育犬頭数推計値 (千頭)

	JFPA	SPBI	SPBP
調整なし (人口ウエイトのみ)	10067	9384	14130
世帯人員ウエイト	8171	7810	12511
傾向スコアウエイト	10044	9705	

図 1　飼育犬頭数分布 (SPBI 世帯人員ウエイト, SPBP 世帯人員ウエイト, SPBI 傾向スコアウエイト)

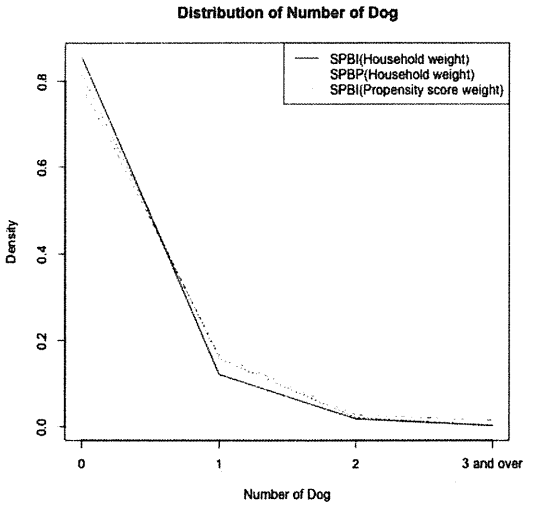
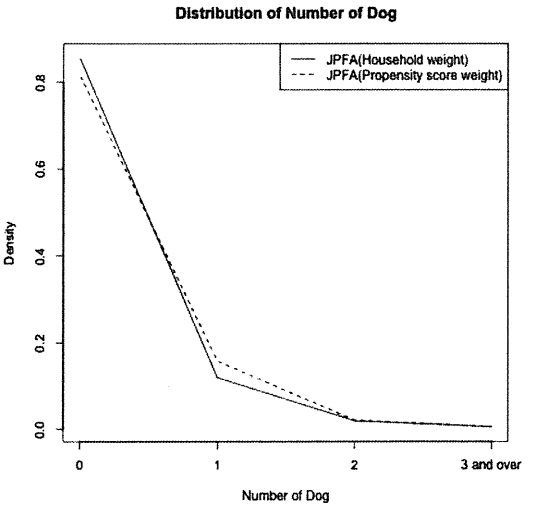


図 2　飼育犬頭数分布 (JFPA 世帯人員ウエイト, JFPA 傾向スコアウエイト)



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研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Two Japanese *Corynebacterium ulcerans* isolates from the same hospital: ribotype, toxigenicity and serum antitoxin titre

Takako Komiya,[1] Yukiji Seto,[2]† Aruni De Zoysa,[3] Masaaki Iwaki,[1] Akio Hatanaka,[4]‡ Atsunobu Tsunoda,[4]§ Yoshichika Arakawa,[1] Shunji Kozaki[2] and Motohide Takahashi[1]

Correspondence
Masaaki Iwaki
miwaki@nih.go.jp

[1]Department of Bacteriology II, National Institute of Infectious Diseases, Tokyo, Japan

[2]Infectious Diseases Control, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Osaka, Japan

[3]Streptococcus and Diphtheria Reference Unit, Respiratory and Systemic Infections Department, Health Protection Agency Centre for Infections, London, UK

[4]Asahi General Hospital, Chiba, Japan

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Two toxigenic *Corynebacterium ulcerans* isolates recovered from pharyngeal swabs of two patients from the same hospital in Japan during 2001–2002 were characterized by PFGE and ribotyping. Toxin production in different culture media was examined and serological analysis of patient sera was performed. The two isolates could not be distinguished by PFGE; however, their ribotypes were distinguishable. One of the isolates could represent a novel ribotype. Analysis of toxin production in different culture media demonstrated that the two isolates produced varying amounts of the diphtheria toxin. Serological analysis showed a greater than sevenfold increase in the serum antitoxin titre during the course of infection in one patient.

INTRODUCTION

Disease caused by *Corynebacterium ulcerans* is now an emerging threat to human health (CDC, 1997; DeWinter *et al.*, 2005; Kisely *et al.*, 1994; Lartigue *et al.*, 2005; PHLS, 2000; Sing *et al.*, 2005; Von Hunolstein *et al.*, 2003), and has recently been classified as diphtheria by the European Centre for Disease Prevention and Control (http://ecdc.europa.eu/en/healthtopics/pages/diphtheria.aspx). In Japan, the Ministry of Health, Labour and Welfare released notifications concerning this disease twice in 2002 and 2009, although the disease is not classified as diphtheria in this country.

C. ulcerans is known as a major cause of mastitis in cows. A significant portion of human *C. ulcerans* infections in the UK has been associated with ingestion of raw dairy products (Galbraith *et al.*, 1982; Hart, 1984; Kisely *et al.*, 1994). This organism has also been recently recognized as a human pathogen associated with companion animals such as dogs and cats (De Zoysa *et al.*, 2005; Dias *et al.*, 2010; Lartigue *et al.*, 2005; SCIEH, 2002; Taylor & Efstratiou, 2002) and pigs (Schuhegger *et al.*, 2009).

The first case of human infection with toxigenic *C. ulcerans* was described in 1970 (Fakes & Downham, 1970) in the UK. In early 2001, 30 years after the first report, the organism was identified as a human pathogen in Japan in a patient who had been feeding 20 cats in and around her home (Hatanaka *et al.*, 2003). The second case in Japan occurred around late 2002, and the two cases occurred in locations about 5 km apart. Both cases were reported from the same hospital. The first case has already been briefly reported (Hatanaka *et al.*, 2003), but there have been no reports of the second case. No detailed characterization of the isolates or analysis of patient sera has been reported for either of the cases.

Diphtheria caused by *Corynebacterium diphtheriae* results in a detectable rise in the serum antitoxin titre against diphtheria toxin during the course of infection (Danilova

†Present address: Tochigi Prefectural Livestock Experiment Station, Tochigi, Japan.

‡Present address: Department of Otorhinolaryngology, Tsuchiura Kyodo General Hospital, Ibaraki, Japan.

§Present address: Department of Otolaryngology, Tokyo Medical and Dental University, Tokyo, Japan.

Abbreviations: CD_{50}, 50 % cytopathic dose; CDC, Centers for Disease Control and Prevention; MRD, minimal reactive dose; PHLS, Public Health Laboratory Service; PLD, phospholipase D; SCIEH, Scottish Centre for Infection and Environmental Health.

A table of primer details and figures of PCR identification and sequence alignment data are available as supplementary material with the online version of this paper.

et al., 2006). We were interested to know whether toxigenic *C. ulcerans* would be capable of inducing a similar reaction. In this study, we present the detailed molecular characteristics of the *C. ulcerans* isolates from the two Japanese cases, and show that a marked increase in serum antitoxin titre occurred during the course of infection in one patient.

METHODS

Bacterial strains, culture media and standard materials. Bacterial strains used in this study are listed in Table 1. Identification of the strains was performed using an API Coryne kit (bioMérieux). The bacterial strains were cultivated on Loeffler medium (Kyokuto Pharmaceutical Industrial), sheep blood agar plates (Nissui Pharmaceutical), in iron-depleted Pope liquid medium (Tasman & Van Ramhorst, 1951; Tchorbanov *et al.*, 2004) or brain heart infusion broth (Difco; Becton Dickinson). Japanese national reference diphtheria test toxin lot M59 was used as a control toxin. Japanese national standard diphtheria antitoxin lot 10, which was calibrated against the World Health Organization international standard antitoxin, was used as a reference material for antitoxin unit definition.

Gram staining and Neisser staining. Gram staining of the bacterial strains was performed using a Gram staining kit (Nissui Pharmaceutical). Neisser staining was performed according to the method originally described by Neisser (Hendrickson & Krenz, 1991).

PFGE typing. PFGE typing of the bacterial strains was performed essentially according to De Zoysa *et al.* (1995). The bacterial strains to be analysed were briefly cultured on sheep blood agar plates overnight at 37 °C. The bacterial cells were then collected, embedded in agarose plugs and lysed overnight at 37 °C using freshly prepared lysis solution (Murray *et al.*, 1990) containing 1 mg lysozyme ml^{-1}. The cells were then treated with proteolysis buffer (Murray *et al.*, 1990) for 48 h at 37 °C. DNA from the cells embedded in the plugs was subsequently digested with *Sfi*I (New England Biolabs) overnight at 37 °C and the digested samples were applied to an analytical agarose gel. PFGE was performed in a CHEF DRII apparatus (Bio-Rad) at 11 °C with a pulse time of 5 to 20 s for the first 20 h, and then 1 to 5 s for the following 18 h.

Ribotyping. Ribotyping of bacterial strains was performed as described by De Zoysa *et al.* (1995) and Regnault *et al.* (1997). Isolated genomic DNA was digested with *Bst*EII (Roche Diagnostics), electrophoresed in an agarose gel and transferred to HyBond Plus nylon membrane (Amersham Biosciences). The transferred DNA was hybridized with a DIG-labelled OligoMix 5 probe mixture (Grimont *et al.*, 2004; Regnault *et al.*, 1997), and signals were detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics). Ribotype profiles were analysed using the BioNumerics software program (version 3.0; Applied Maths).

PCR and nucleotide sequence determination. Genomic DNA was isolated from the bacterial strains using a blood mini kit (Qiagen) according to the kit instructions. PCR detection of the diphtheria toxin (*tox*) gene was performed by amplifying a 248 bp fragment located in the middle of the catalytic domain (A subunit) of the gene using oligonucleotide primers Tox 1 and Tox 2 (Supplementary Table S1 available with the online journal) (Mikhailovich *et al.*, 1995). The phospholipase D (PLD)-encoding gene was detected using primers CorynePLD-F and CorynePLD-R1 (Supplementary Table S1 available with the online journal).

The nucleotide sequence of the *tox* gene was determined by amplifying five fragments covering the entire ORF (spanning a region upstream as well as downstream of the *tox* gene) using the primer pairs of DT-1 and Tox 1, Tox 2 and DT-2, DT-3 and Dipht 6R, Dipht 6F and DT-4, and DT-5 and DT-6 (Supplementary Table S1 available with the online journal) (Mikhailovich *et al.*, 1995; Nakao *et al.*, 1996). Nucleotide sequences of the amplified fragments were determined by cycle sequencing using a BigDye terminator kit (Applied Biosystems) and analysed using an Applied Biosystems model 310 genetic analyser.

Toxigenicity assays. Immunological detection of diphtheria toxin was performed using a further modification of the modified Elek method (Reinhardt *et al.*, 1998). A 9 mm well was made aseptically on an agar plate containing 20 g proteose peptone l^{-1} (Becton Dickinson), 1 g Bacto yeast extract l^{-1} (Becton Dickinson), 2.5 g NaCl l^{-1}, 20 % (v/v) newborn calf serum (Gibco; Life Technologies) and 15 g Bacto agar l^{-1} (Becton Dickinson). The bacterial strains to be tested were then inoculated at a distance of 10 mm from the edge of the well, which was then filled with an aqueous solution containing 4.5 units of standard diphtheria antitoxin. The precipitin lines formed were observed after 1 to 2 days of cultivation at 37 °C.

For Vero cell and rabbit skin toxigenicity tests (Miyamura *et al.*, 1974a, b), the liquid condensed at the base of the Loeffler slant ('Loeffler condensation') and the culture supernatant of the Pope liquid medium ('Pope culture supernatant') were sterile filtered and used as specimens. Serially diluted specimens were added to Vero cells seeded in 96-well culture plates, and the neutralization end points were determined by observing cytotoxic effects 4 days post-incubation, as described by Miyamura *et al.* (1974a, b). Standard antitoxin

Table 1. Bacterial isolates and strains used in this study

Isolate and strain no.	Patient source			Clinical symptom	Reference
	Sex	Age	Site of isolation		
Corynebacterium ulcerans 0102	F	52	Throat (pseudomembrane)	Dyspnoea, fever	Hatanaka *et al.* (2003)
Corynebacterium ulcerans 0211	M	54	Throat (pseudomembrane)	Sore throat, fever	This study
Corynebacterium ulcerans ATCC 51799	–	–	–	–	ATCC
Corynebacterium diphtheriae PW8	–	–	–	–	Laboratory stock
Corynebacterium diphtheriae ATCC 700971	–	–	–	–	ATCC (NCTC 13129)
Rhodococcus equi ATCC 6939	–	–	–	–	ATCC
Citrobacter koseri CIP 105177	–	–	–	–	CIP

ATCC, American Type Culture Collection; CIP, Collection de l'Institut Pasteur; F, female; M, male; NCTC, National Collection of Type Cultures; PW8, Park–Williams no. 8.

(40 IU ml^{-1}) was used to confirm that the toxicity was due to diphtheria toxin.

For the rabbit skin toxigenicity test, specimens were appropriately diluted in PBS containing 0.2 % gelatin, and 100 µl of this solution were intradermally injected into the shaved back of a female Japanese white rabbit weighing 3.5 kg. The diameter of local erythema formed was measured on day 2 post-injection. Standard antitoxin (1 IU ml^{-1}) was used to confirm that the toxicity was due to diphtheria toxin. Animal experiments were performed with the approval of the Animal Experiment Committee of the National Institute of Infectious Diseases. PLD activity was visualized by enhanced haemolysis on sheep blood agar plates when *C. ulcerans* isolates and *Rhodococcus equi* ATCC 6939 were streaked close to each other on the plates.

Serum antitoxin titre determination. Anti-diphtheria toxin neutralization titre was determined by the Vero cell cytotoxicity assay described above. Serially diluted patient serum or standard antitoxin was mixed with 16 CD_{50} ml^{-1} [i.e. (16 times the CD_{50}) ml^{-1}] of the reference diphtheria test toxin and added to the Vero cell cultures (Miyamura *et al.*, 1974a). The antitoxin titre of patient sera was determined by comparing the neutralization end points of the patient sera and standard antitoxin, and was expressed in IU.

RESULTS

Cases

The first case has been reported previously (Hatanaka *et al.*, 2003). Briefly, the patient was a 52-year-old woman who presented to the Asahi General Hospital in Chiba Prefecture, Japan, on 16 February 2001, with dyspnoea and a sore throat. *C. ulcerans* was isolated from a pharyngeal pseudomembrane. She recovered without any serious sequelae following erythromycin treatment. The strain 0102 was isolated from this patient.

The second case was a 54-year-old man who presented to the same hospital with a sore throat and fever on 28 October 2002. *C. ulcerans* was isolated from a yellow-white pseudomembrane observed in his pharynx and was designated 0211. He recovered without any serious sequelae following clarithromycin treatment. No contact with dairy livestock, raw dairy products or domestic animals was reported.

Identification of the isolates

Bacteriological characteristics of the two *C. ulcerans* isolates are summarized in Table 2. Both strains exhibited Gram-positive short rod morphology on Gram staining. Clearly distinguishable metachromatic granules were observed at the ends of the cells by Neisser staining (Hendrickson & Krenz, 1991). Both strains were biochemically characterized as *C. ulcerans* with a probability of 99.7 % using the API Coryne kit (code 0111326).

Genotyping of the isolates

PFGE patterns are shown in Fig. 1(a). The PFGE patterns of isolates 0102 and 0211 are indistinguishable, whereas the pattern for *C. ulcerans* ATCC 51799 is clearly different from that for the Japanese isolates. The ribotype profiles of the two isolates are shown in Fig. 1(b), and comparison by cluster analysis of these profiles with that of the *C. ulcerans* ribotypes in an in-house database (De Zoysa *et al.*, 2005) revealed that the ribotype profile of isolate 0102 showed 100 % similarity with ribotype U4, which is a predominant profile seen among human clinical *C. ulcerans* isolates in the UK. The ribotype profile of isolate 0211 did not match with any of the known *C. ulcerans* ribotypes (U1–U9), suggesting that it could potentially be a new ribotype (Fig. 1c).

Toxigenicity tests

The presence of the *tox* gene in both isolates was demonstrated by PCR using oligonucleotide primers Tox 1 and Tox 2 (Supplementary Table S1 available with the online journal) (Mikhailovich *et al.*, 1995) to detect a 248 bp amplified fragment corresponding to the middle of the catalytic domain (A subunit) of the diphtheria toxin gene. The *tox* gene was detected in both strains (Table 2).

Table 2. Bacteriological characteristics of the isolates

Test	Isolate	
	0102	0211
Gram staining	Gram positive	Gram positive
Metachromatic granules by Neisser staining	+	+
Identification by API Coryne kit	*C. ulcerans* (99.7 %; code 0111326)	*C. ulcerans* (99.7 %; code 0111326)
Toxigenicity		
tox gene PCR	+	+
Elek assay	+	ND
PLD		
pld gene PCR	+	+
R. equi-stimulated haemolysis	+	+

ND, Not detectable.

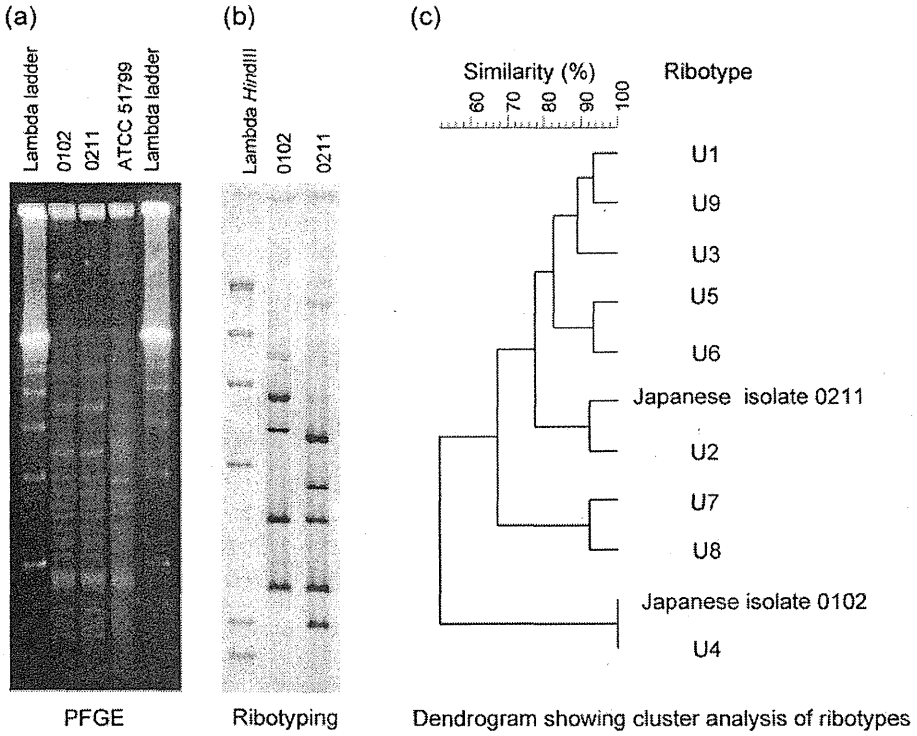

Fig. 1. PFGE and ribotype patterns of the isolates. (a) PFGE patterns. Genomic DNA of *C. ulcerans* clinical isolates 0102 and 0211 and of *C. ulcerans* ATCC 51799 was digested with *Sfi*I and subjected to PFGE as described in Methods. Lambda phage DNA ladders were used as molecular mass markers. (b) Ribotype patterns of the clinical isolates. Genomic DNA of *C. ulcerans* clinical isolates 0102 and 0211 were digested with *Bst*EII and subjected to agarose gel electrophoresis and Southern blotting as described in Methods. Lambda *Hin*dIII-digested DNA was used as a molecular mass marker. (c) Cluster analysis of ribotypes. Similarity between ribotyping band patterns of the clinical isolates were compared with those of nine known ribotypes (U1–U9), which were calculated according to the UPGMA method.

The sizes of the amplified fragments were indistinguishable from each other and from a DNA fragment amplified from genomic DNA of *C. diphtheriae* vaccine strain PW8 by agarose gel electrophoresis (data not shown).

Immunological detection of diphtheria toxin was performed using the modified Elek method (Reinhardt *et al.*, 1998). A precipitin line was observed for isolate 0102 but isolate 0211 did not exhibit a clearly observable precipitin line on the Elek plate, suggesting that the amount of toxin produced by isolate 0211 in this culture medium was less than that produced by isolate 0102.

The activity of the toxin produced by the isolates was then measured using Vero cell and rabbit skin toxigenicity tests according to Miyamura *et al.* (1974a). Loeffler condensation of isolate 0102 exhibited 513 CD_{50} ml^{-1} of cytotoxicity based on the Vero cell test, and the toxicity was completely neutralized by 40 IU ml^{-1} (1 IU per culture well) of diphtheria antitoxin (Table 3). In contrast, Loeffler condensation of isolate 0211 showed approximately twofold less cytotoxicity (208 CD_{50} ml^{-1}) but neutralization by the same concentration of antitoxin was only partial, retaining 114 CD_{50} ml^{-1} (Table 3). Because *C. diphtheriae* vaccine strain PW8 showed 2×10^4 CD_{50} ml^{-1} and this activity was completely neutralized by the same concentration of antitoxin, the partial neutralization observed for 0211 was not thought to be caused by insufficient serum antitoxin titre.

Toxin activity was also detectable by the rabbit skin toxigenicity test (Miyamura *et al.*, 1974a). Loeffler condensation of isolate 0102 contained 80 MRD ml^{-1} [i.e. (80 times the MRD) ml^{-1}] of activity. In contrast to the results from the Vero cell assay, 20 MRD ml^{-1} of this activity remained unneutralized after treatment with 1 IU standard diphtheria antitoxin ml^{-1} (Table 3). The condensation of isolate 0211 showed less activity (20 MRD ml^{-1}), which was neutralized incompletely by the antitoxin (Table 3).

C. ulcerans strains are known to produce another toxic factor PLD. The presence of the PLD-encoding gene and secretion of the active enzyme (visualized by cross-streaking with *R. equi* ATCC 6939) occurred in both isolates (Supplementary Fig. S1 available with the online journal). PLD may be an additional factor contributing to

Table 3. Toxicity of *C. ulcerans* assessed by Loeffler condensation and Pope culture supernatant

Assays were done in duplicate and repeated three times (Vero cytotoxicity) or once (rabbit erythema).

	Loeffler condensation			Pope culture supernatant		
	C. ulcerans		*C. diphtheriae*	*C. ulcerans*		*C. diphtheriae*
	0102	0211	PW8	0102	0211	PW8
Vero cytotoxicity (CD_{50} ml^{-1})						
Before neutralization	513 ± 238*	208 ± 50*	2.0×10^4†	640†	$1.4 \pm 0.006 \times 10^4$*	$3.9 \pm 0.02 \times 10^5$*
After neutralization‡	ND	114 ± 17*	ND	ND	ND	ND
Rabbit erythema assay (MRD)						
Before neutralization	80 (40, 160)§	20‖	2.0×10^4‖	640‖	1.6×10^4 (8.0×10^3, 3.2×10^4)§	2.0×10^4‖
After neutralization‖	20¶	10¶	ND	ND	ND	ND

ND, Not detectable.
*Mean ± SE.
†The same results were obtained in three repeats.
‡Neutralization with 40 IU diphtheria antitoxin ml^{-1}.
§Geometric mean of a duplicate assay. Lower and higher values are indicated in parentheses.
‖The same results were obtained in a duplicate assay.
¶Neutralization with 1 IU diphtheria antitoxin ml^{-1}.

unneutralized cytotoxicity of Vero cells and to erythema formation on rabbit skin.

Nucleotide sequences of the *tox* genes

We then determined the nucleotide sequence of five fragments covering the entire *tox* gene and its flanking regions by amplifying these with the primer pairs shown in Supplementary Table S1 (available with the online journal) (Mikhailovich *et al.*, 1995; Nakao *et al.*, 1996). The alignment of the nucleotide sequences of the *tox* genes in *C. ulcerans* isolates 0102 and 0211, and *C. diphtheriae* PW8, is shown in Supplementary Fig. S2 (available with the online journal). The sequences from isolates 0102 (GenBank/EMBL/DDBJ accession no. AB304278) and 0211 were identical to each other and to that of the German *C. ulcerans* isolate A6361 (Sing *et al.*, 2003). In addition, the nucleotide sequence of the upstream region, as determined by sequencing of DNA fragments amplified with primers DT-1 and Tox 1 (Supplementary Table S1 available with the online journal), were identical to each other.

The *tox* gene of isolates 0102 and 0211showed a difference of 81 nt (corresponding to 27 amino acids) compared with that of the strain PW8 (Supplementary Fig. S2 available with the online journal). The major difference between the *C. ulcerans* toxin and PW8-derived toxin was observed in the receptor-binding domain in the B subunit of the toxin molecule.

Dependence of toxin production on culture medium

In contrast to the identity in *tox* gene sequences, the two isolates differed in terms of the effect of culture conditions on toxin production. Toxin production by the two *C. ulcerans* isolates on the Loeffler medium and in iron-depleted Pope liquid medium, a medium frequently used for diphtheria toxin production (Tasman & Van Ramhorst, 1951; Tchorbanov *et al.*, 2004), were compared (Table 3). A large difference was observed between the two *C. ulcerans* isolates in their response to culture media (Table 3). Strain 0211 exhibited a more than 70-fold difference in Vero cell cytotoxicity when cultured in the different media (208 CD_{50} ml^{-1} for Loeffler condensation and 14 000 CD_{50} ml^{-1} for Pope culture supernatant). In contrast, strain 0102 showed a less than twofold difference in cytotoxic activity between the Loeffler medium and Pope medium (513 CD_{50} ml^{-1} for Loeffler condensation and 640 CD_{50} ml^{-1} for Pope culture supernatant), suggesting that the two strains do not share a common mechanism for regulating toxin activity. The strains also differed in the rabbit skin toxigenicity test. Strain 0211 exhibited an erythema forming activity at 20 MRD when cultured on Loeffler medium and 800-fold more activity (1.6×10^4 MRD ml^{-1}) in Pope medium; these results are comparable to that of *C. diphtheriae* vaccine strain PW8. In contrast, strain 0102 showed only an eightfold difference in its ability to induce erythema formation (80 vs 640 MRD ml^{-1} on Loeffler medium and Pope medium, respectively).

The product of the *dtxR* gene is an important factor regulating *C. diphtheriae* diphtheria toxin gene expression in response to the iron content in culture media (Boyd *et al.*, 1990). In the two *C. ulcerans* strains, the *dtxR* gene was detectable by Southern blotting (Fig. 2). In strain 0102, the nucleotide sequence of the *dtxR* gene (including its 5′- and 3′-flanking regions) amplified with the primers listed in Supplementary Table S1 (available with the online journal) was identical to reported sequences (data not

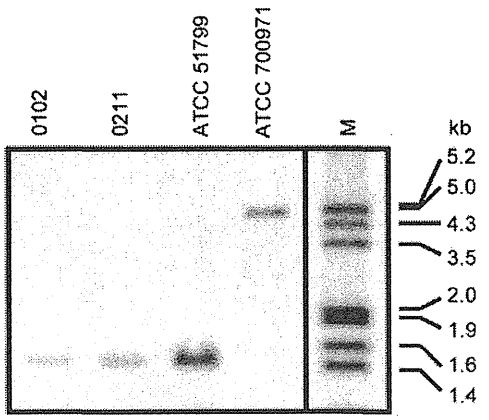

Fig. 2. Presence of the *dtxR* gene in the clinical isolates. Genomic DNA from *C. ulcerans* 0102 and 0211, ATCC 51799 and *C. diphtheriae* ATCC 700971 (equivalent to NCTC 13129) was digested with *Hin*dIII and was subjected to Southern blotting and detected using a DIG-labelled *dtxR* probe. M represents lambda *Eco*RI–*Hin*dIII digested DNA, which was used as a molecular mass marker.

shown) (Boyd *et al.*, 1990). However, for strain 0211, amplification of the region was not possible, suggesting a difference in nucleotide sequences in at least one of the primer regions flanking the *dtxR* gene, which could affect the isolate's toxin production in response to culture conditions.

Serum antitoxin titre

Respiratory infection with toxigenic *C. diphtheriae* is known to cause elevation of serum antitoxin during the course of infection (Danilova *et al.*, 2006); however, it is not yet clear if *C. ulcerans* respiratory infection can cause such an elevation. From the second case reported in our study (in which strain 0211 was isolated), patient serum was obtained periodically from the time of first presentation to the hospital on 28 October 2002 until 16 February 2005. The diphtheria antitoxin titre was measured by Vero cell neutralization assay (Miyamura *et al.*, 1974a) using Japanese national standard diphtheria antitoxin lot 10 as a reference (Table 4). The vaccination history of the patient was unclear. At first presentation to the hospital, the serum antitoxin titre was undetectable. However, the antitoxin titre started to increase 2 weeks thereafter (0.39 IU ml^{-1}) and rapidly rose to almost 2.5 IU ml^{-1} within a further 3 weeks. The high antibody titre was retained until at least the last sampling date (more than 2 years later). The fully protective level of antitoxin titre against diphtheria is believed to be 0.1 IU ml^{-1} (Galazka, 1993; Hasselhorn *et al.*, 1998). This result indicates that human respiratory *C. ulcerans* infection can induce an antibody response against diphtheria toxin, as well as in the reported cases of *C. diphtheriae* infection (Danilova *et al.*, 2006), and that the rise in antibody titre can be utilized as a marker of *C. ulcerans* infection.

Table 4. Serum antitoxin titre from the second case

All assays were done in triplicate and repeated three times, twice or once.

Date	Titre (IU ml^{-1})
28 October 2002	ND
11 November 2002	0.39±0.06*
2 December 2002	2.47±0.00*
14 April 2003	2.72±0.05*
24 December 2003	1.18†
16 February 2005	0.92‡

ND, Not detectable.
*Values are the mean±SE; assays were repeated three times.
†Repeated assays gave the same results; assays were repeated twice.
‡The same results were obtained in triplicate; assays were repeated once.

DISCUSSION

In the present study, two toxigenic *C. ulcerans* isolates recovered from pharyngeal swabs were characterized. The two isolates were indistinguishable by PFGE but could be distinguished by ribotyping. The isolate 0211, isolated in 2002, could potentially represent a new ribotype that has not been seen in Europe or North America. Geographically specific distribution has already been reported for some *C. ulcerans* ribotypes (De Zoysa *et al.*, 2005). Whether this new ribotype is specific to the Asian region or not will be clarified with further collection of clinical isolates from this region.

In the 2002 case, the patient showed a marked increase in serum antitoxin titre during the course of infection, which is, to our knowledge, the first evidence for such an increase in a *C. ulcerans* respiratory case. The patient showed a remarkable increase in titre within 2 weeks after he had first presented to the hospital, and the elevated titre remained for more than 2 years at least. Whether this rapid and lasting antibody response was based on previous vaccination is not clear, because insufficient information was available on the vaccination history of the patient. An increase in serum antitoxin titre during cutaneous infection caused by *C. ulcerans* has also been reported by Wagner *et al.* (2001).

Analysis in different culture media showed that the two isolates exhibited varying degrees of toxin activity (Table 3). First, for both isolates the toxin activities in Loeffler condensation were not completely neutralized by diphtheria antitoxin. The deduced amino acid sequence of their *tox* gene product was different from that of *C. diphtheriae* PW8 by 28 amino acid residues, mainly in the receptor-binding domain of the toxin molecule. However, it is not likely that the difference could account for the incomplete neutralization. When cultured in Pope liquid medium,

both isolates showed higher toxin activity than in Loeffler condensation, and the activity was completely neutralized by the same antitoxin. This suggests that, in addition to the *tox* gene product, on Loeffler medium the *C. ulcerans* isolates could secrete toxic substances immunologically discrete from the *tox* gene product, including PLD. In contrast, in Pope medium, the major part of toxicity could be attributed to their *tox* gene products.

Another feature varying between the two isolates was the response to culture conditions. The 0211 isolate responded well to the change of culture conditions. In contrast, the response of 0102 was not remarkable. This indicates that the isolates do not share the same mechanism for the regulation of toxin activity, even though the nucleotide sequence of the upstream region of the *tox* gene was identical in these isolates, as determined by the sequencing of DNA fragments obtained by PCR with primers DT-1 and Tox 1.

Concerning the regulatory gene *dtxR*, the presence of the gene was demonstrated in both isolates by the Southern hybridization experiment. However, for isolate 0211, the nucleotide sequence of the gene could not be determined because amplification of DNA fragments spanning from the upstream region to the N-terminal region of the ORF was impossible with primers dtxRprimerF#6 and dtxRprimerR#2 (Supplementary Table S1 available with the online journal) designed based on the reported nucleotide sequence (Boyd *et al.*, 1990). Within this region there might be some significant difference in nucleotide sequence that remains to be elucidated, which affects the regulation of *tox* gene regulation.

Mass immunization against diphtheria, as well as tetanus and pertussis, has been conducted in many countries for decades with diphtheria toxoid. With the increasing importance of *C. ulcerans* human infection, immunization with toxoid and therapy with diphtheria antitoxin are considered to be effective against the disease (CDC, 1997; De Zoysa *et al.*, 2005; Tiwari *et al.*, 2008; Von Hunolstein *et al.*, 2003). In the present paper we have shown that serum from a patient was able to neutralize diphtheria toxin at a high titre. Our data also suggest that cross protection between *C. diphtheriae* and *C. ulcerans* infections would be possible.

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Novel *Corynebacterium diphtheriae* in Domestic Cats

Aron J. Hall, Pamela K. Cassiday, Kathryn A. Bernard, Frances Bolt, Arnold G. Steigerwalt, Danae Bixler, Lucia C. Pawloski, Anne M. Whitney, Masaaki Iwaki, Adam Baldwin, Christopher G. Dowson, Takako Komiya, Motohide Takahashi, Hans P. Hinrikson, and Maria L. Tondella

Novel nontoxigenic *Corynebacterium diphtheriae* was isolated from a domestic cat with severe otitis. Contact investigation and carrier study of human and animal contacts yielded 3 additional, identical isolates from cats, although no evidence of zoonotic transmission was identified. Molecular methods distinguished the feline isolates from known *C. diphtheriae*.

The clinical relevance of *Corynebacterium diphtheriae* recovered from a cat with otitis is poorly understood. Historically, humans have been thought to be its sole reservoir, and the few human cases reported annually in the United States are generally associated with international travel (*1*). Therefore, when *C. diphtheriae* was isolated from the ears of a cat, an investigation was initiated to evaluate potential sources of the cat's infection and potential public health risks and to preliminarily characterize the *C. diphtheriae* isolate.

The cat, an 8-month-old female domestic shorthair, was examined at a West Virginia veterinary hospital on 5 occasions during January–June 2007. Pertinent findings included severe bilateral otitis, vestibular signs, mild ataxia, anorexia, and failure to gain weight; the cat had a history of ear, eye, and lung infections. Results of diagnostic tests showed no evidence of systemic disease and were negative for feline immunodeficiency and leukemia viruses and feline infectious peritonitis. Culture of an otic swab collected from the cat in May 2007 yielded 4 organisms: *C. diphtheriae*, *Streptococcus equi zooepidemicus*, *Staphylococcus* spp., and *Achromobacter xylosoxidans*. The cat was treated with oral clindamycin, otic enrofloxacin, and an ear-flushing solution.

Author affiliations: Centers for Disease Control and Prevention, Atlanta, Georgia, USA (A.J. Hall, P.K. Cassiday, A.G. Steigerwalt, L.C. Pawloski, A.M. Whitney, H.P. Henrikson, M.L. Tondella); West Virginia Department of Health and Human Resources, Charleston, West Virginia, USA (A.J. Hall, D. Bixler); Public Health Agency of Canada, Winnipeg, Manitoba, Canada (K.A. Bernard); University of Warwick, Coventry, UK (F. Bolt, A. Baldwin, C.G. Dowson); and National Institute of Infectious Diseases, Tokyo, Japan (M. Iwaki, T. Komiya, M. Takahashi)

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The Study

In June 2007, investigators visited the veterinary clinic and the household of the index cat and conducted a contact investigation and carrier study. Interviews of 2 household members and 8 veterinary staff members indicated no recent respiratory illness, skin infection, or risk factors for diphtheria (e.g., travel to countries to which diphtheria is endemic or contact with known case-patients). Half of these 10 contacts had received diphtheria vaccination within the previous 5 years. Cultures of oropharyngeal swab samples obtained from each person were negative, including cystine tellurite blood agar, which is selective for *C. diphtheriae*. Household members also were interviewed about medical history of a convenience sample of household animals (4 cats, including the index cat; 2 dogs; and 1 horse). Each animal was briefly examined, and oropharyngeal, otic, or ocular swab samples were collected. Otitis was observed in all 4 cats and 1 dog. The horse reportedly had had an eye infection ≈5 years earlier. No other abnormal findings were noted. Animal specimens yielded 3 additional isolates of *C. diphtheriae*: 1 from each ear of the index cat and 1 from the left ear of a 2-year-old domestic medium-hair cat. Both cats had been born on the premises and had remained with the same household since birth.

Feline *C. diphtheriae* and reference isolates used are described in the Table. Tinsdale agar plate growth (Remel, Lenexa, KS, USA) gave rise to black colonies with a brown halo, typical of cysteinase-producing *C. diphtheriae*, *C. ulcerans*, or *C. pseudotuberculosis*. After 24 hours on blood agar, 1–2-mm grey-white or opaque, rounded, convex colonies with no hemolysis were observed. Microscopically, the bacteria were gram-positive, club-shaped rods, 1 μm in diameter, arranged singly or at angles. Biochemical profiles to determine species and biotype were done by using an API Coryne strip (bioMérieux, Durham, NC, USA, and St-Laurent, Quebec, Canada). Query of API Coryne code 0010304 obtained for all isolates by APIWEB (https://apiweb.biomerieux.com) indicated a decreased level of confidence of *C. diphtheriae* biotype *mitis* or *belfanti* (89.5%) because of a maltose-negative result. Isolates were further characterized morphologically and biochemically by using tube substrates (*2*) and were identified by using a standard taxonomic scheme (*3*). Feline isolates were biochemically identical with each other and phenotypically consistent with *C. diphtheriae* biotype *belfanti*, except for the lack