表 5. 食品の細菌学的成分規格および試験法一覧表（食鳥卵）

	食品	サルモネラ属菌	細菌数
60	食鳥卵 未殺菌液卵（殺菌液卵以外の鶏の液卵）	/	100 万/g 以下
61	食鳥卵 殺菌液卵（鶏の液卵を殺菌したもの）	陰性/25 g	/

検査項目	培養		試料の調整	
	温度 (℃)	培地（時間）	試料の希釈倍率と培地の数	希釈液
細菌数	35±1.0	PC (24±2)	×1, ×10, ×100, ×1,000; 1 m*l*×2 枚[4]	希釈液の規定なし
サルモネラ属菌	36±1	mBPW[1] (22±2)	25 g+225 m*l* (mBPW)	
	42±0.5	TT, 10 m*l* (22±2) RV, 10 m*l* (22±2)	mBPW の 0.5 m*l*	
	36±1	MLCB[2] (18～24) BGS[3] (18～24)	TT, RV の各 1 エーゼ	
	36±1	TSI, LIM あるいは LIA (18～24)		
		血清学的試験および生化学的試験を行い同定する（同定キットも使用可）		

[1] mBPW: L-システイン 0.2 g/*l* 又は $FeSO_4 \cdot 7H_2O$ を 64 mg/*l* に添加した BPW
[2] 硫化水素産生により判定する培地で MLCB, DHL, XLD 等
[3] 硫化水素非産生であってもサルモネラと判定できる培地で BGS, BGM（改良 BGA），ランバック培地，SMID 等
[4] 氷雪と同様に実施することが指示されている．そのまま解釈すれば採取試料量は原液 (1 m*l*) からとなる．

表 6. 食品の細菌学的成分規格および試験法一覧表（ゆでだこ等の腸炎ビブリオ）

食品	細菌数	大腸菌群	腸炎ビブリオ（定性）	腸炎ビブリオ (MPN)
62 （非冷凍）ゆでだこ	/	/	陰性	/
40 冷凍ゆでだこ	10 万/g 以下	陰性 (DS)	陰性	/
喫食時に加熱を要しない				
63 （非冷凍）ゆでがに	/	/	陰性	/
41 冷凍ゆでがに	10 万/g 以下	陰性 (DS)	陰性	/
喫食時に加熱を要す[1]				
64 冷凍ゆでがに	10 万/g 以下	陰性 (DS)	/	/
65 生食用鮮魚介類[2]	/	/	/	100/g 以下
46 むき身にした生食用かき	5 万/g 以下	E. coli, 230/100 g 以下	/	100/g 以下
39 生食用冷凍鮮魚介類	10 万/g 以下	陰性 (DS)	/	100/g 以下

検査項目	培養		試料の調整
	温度 (℃)	培地（時間）	試料の希釈倍率と培地の数
腸炎ビブリオ（定性）	37	2%NaCl 加 AP（一夜培養）	25 g+225 m*l*
	37	TCBS（一夜培養） 集落を同定し判定	1 白金耳
腸炎ビブリオ (MPN)	37	2%NaCl 加 AP, 10 m*l*（一夜培養）	25 g+225 m*l*（3%NaCl 加 PB)—×10 ↓1 m*l* 9 m*l*（3%NaCl 加 PB)—×100 (×10; 1 m*l*, ×100; 1 m*l*, 0.1 m*l*)×3 本
	37	TCBS（一夜培養） 集落を同定し判定	1 白金耳

AP: アルカリペプトン水 (pH 8.6)，3%NaCl 加 PB: 生食用かきの検査に用いる PB に 3% の NaCl を加えたもの．
[1] 喫食時に加熱を要す（非冷凍）ゆでがにには規格はない．
[2] 生食用鮮魚介類：切り身又はむき身にした鮮魚介類（生かきを除く）であって，生食用のもの（凍結させたものを除く）に限る．
注) 定性試験の培養及び判定，MPN 試験における最確数の算定については，同等以上の性能を有すると認められる方法で行うことが認められた．なお，冷凍ゆでだこ，冷凍ゆでがに，生食用冷凍鮮魚介類の細菌数および大腸菌群の試験法，むき身にした生食用かきの細菌数および E. coli の試験法は別途記載している．

平成5年の食肉製品の規格基準の改訂に際して，成分規格から微生物規格に係る試験法が削除された．これは『微生物試験について日々新しい試験方法が開発されていることに鑑み，新たに開発される試験方法に柔軟に対応するためである』と説明された．実際に平成10年の殺菌液卵の成分規格が告示された際も，サルモネラ試験法は成分規格から除外された．ところが腸炎ビブリオ試験法の規格基準の一部改正（平成13年6月7日）では，上述の理念に反して告示法として成分規格に含まれてしまった．また昭和38年に通知された「病原性好塩菌食中毒検査要項」に記載された腸炎ビブリオ試験法は今でも効力は失われていないのも奇異である．

一覧表から明白なように，試験法の全体的な問題点としては以下のものが挙げられる．①標準寒天培地の培養温度が，乳等省令では32～35℃と幅があるのに対し，その他の食品では35±1.0℃と厳密に規定されている．ところが，両方の温度条件を満たす温度帯がわずかに異なるため，これらの食品を同一培養器では試験できない．②4種類の希釈液を使い分けて食品乳剤を作製しなければならないが，その一方では希釈液が指定されていない食品もある（番号1～7の牛乳類，26のクリーム，27の乳飲料，49～51の氷雪等と清涼飲料水，60の未殺菌液卵）．このような複雑な希釈液の使い分けは，検査者に無用のストレスを与え，検査ミスを誘発する一因にならないとは限らない．③食品の10倍乳剤を作製する際，ほとんどの場合10 g (ml) の食品に希釈液を加えて100 mlにするが，アイスクリーム類や氷菓では90 mlの希釈液を加えることになっている．冷凍食品では食品25 gに希釈液を225 ml，カキでは200 g以上を採取して等量の希釈液を加えることになっている．④これ以外にも，混釈培養する際の培地の温度は43～45℃に保持すると規定されているが，この温度では培地が凝固してしまうために事実上実施不可能である．

5. 検体の採取と試料原液作製の問題点

上記試験法の前段階として，検体の採取・運搬法も細かく規定されている．検体を秤量し決められた希釈液を加えた後にホモジナイズする方法として，手で振る，細砕する，ストマッカー処理等を行うことが食品ごとに厳密に定められている．これらの作業手順は食品衛生小六法で確認する必要はあるが，代表的なものを抜粋して原文のまま記載した．

5.1 乳及び乳製品

牛乳，特別牛乳，殺菌山羊乳，成分調整牛乳，低脂肪牛乳，無脂肪牛乳，加工乳，クリーム及び乳飲料：容器包装のまま採取するか，又はその成分規格に適合するかしないかを判断できる数量を滅菌採取器具を用いて無菌的に滅菌採取瓶に採る．濃縮乳及び脱脂濃縮乳は約200 gを採取する．この場合4度以下の温度で保持し運搬する．検体はその後4時間以内に試験に供しなくてはならない．4時間を超えた場合には，その旨を成績書に付記しなければならない．

濃縮乳及び脱脂濃縮乳にあっては滅菌採取瓶のまま，25回以上よく振り，滅菌スプーンで検体10 gを共栓三角フラスコ（栓を除いて重量85 g以下で100 mlのところにかく線を有するもの）に採り，滅菌生理食塩水を加え100 mlとして10倍希釈液をつくり，以下牛乳，特別牛乳，殺菌山羊乳，成分調整牛乳，低脂肪牛乳，無脂肪牛乳，加工乳，クリームおよび乳飲料と同様に希釈液をつくる．

5.2 冷凍食品

冷凍したまま容器包装の表面をアルコール綿でよくふき，滅菌した器具を用いて開封し，その内容の全体を細切りした後無作為に25 gを無菌的にホモジナイザーに採り，滅菌リン酸緩衝希釈水225 mlを加えて細砕する．

5.3 食肉製品，鯨肉製品及び魚肉ねり製品

微生物試験に供する試料の調整は，製品（スライスハム等細切された製品は除く）の切断すべき表面をアルコール綿でよくふいた後，滅菌した器具を用いて無菌的に切断し，その断面の中央部から25 gを無菌的に採り試料とする．試料に滅菌ペプトン加生理食塩水225 mlを加えて細砕し，試料液とする．

スライスハム等細切された製品にあっては25 gを無菌的に切断して採り試料とする．試料に滅菌ペプトン加生理食塩水225 mlを加えて細砕し，試料液とする．

これらは食品衛生法の成分規格に記載された試験法の一部であるが，例えば以下のように現実には対応することが困難な記載もある．①食肉・魚肉ねり製品等では25 gを中心部から採取するという，他の食品にはない特別な採取法を指定している．中心部から採取しなければならない理由が明確ではなく，しかも平天や竹輪では事実上不可能である．これは実際に問題となった事例である．②現在ほとんどの検査所(室)・研究所で使用しているのはホモジナイザーではなくストマッカーである．③乳および乳製品や氷菓は検体採取後4時間以内に検査することになっているが，実行が困難な場合が多い．④冷凍食品を冷凍したままその全量を細切するのは困難かつ苛酷な作業である．⑤濃縮乳及び脱脂濃縮乳を採取する際に，重量などを厳密に規定した共栓三角フラスコに採取しなければならない理由が明確でない．

6. 混乱しているサルモネラ試験法

既述のような平成5年の食肉製品の規格基準の改訂での方針に従い，平成10年に殺菌液卵の規格基準が告示された際には，サルモネラ試験法は成分規格から除外され別途通知された．しかし，過去の試験法との調和が計られることはなく，それ以外にも2種類のサルモネラ試験法が通知された（表7）．この表で最も注目されるのは，生食用食肉と殺菌液卵のサルモネラ試験法はいずれも平成10年に通知されたにもかかわらず根本的に異な

表7. 食品ごとに異なるサルモネラ試験法

	食肉製品（H5年） 生食用食肉（H10年）	接種量/ 培地量	殺菌液卵（H10年）	接種量/ 培地量	汚染実態調査（H15年）	接種量/ 培地量
試　料	食肉製品：中心部より 25 g 採り細切 生食用食肉：表面を 5×5×1 cm を削り取り， そのうちの 25 g を 1 検体 とする		25 g を混和		25 g をストマッカー処理 15 秒間 または揉み洗いを 20 回程度 （ホモジナイザー処理不可）	
1 次増菌	EEM 225 m*l* （35.0±1.0℃, 18±2 時間）		mBPW[1] 225 m*l* （36±1℃, 22±2 時間）		BPW 225 m*l* （36±1℃, 20～24 時間）	
2 次増菌	SBG, セレナイトまたは TT （43.0±1.0℃ または 35.0±1.0℃, 20±2 時間）	1 m*l*/ 15 m*l*	TT （42±0.5℃, 22±2 時間） RV （42±0.5℃, 22±2 時間）	0.5 m*l*/ 10 m*l* 0.5 m*l*/ 10 m*l*	TT （42±0.5℃, 20～24 時間） RV （42±0.5℃, 20～24 時間）	0.5 m*l*/ 10 m*l* 0.1 m*l*/ 10 m*l*
分離培養	MLCB または DHL （35.0±1.0℃, 24±2 時間）	1 白金耳	MLCB 等[2] （36±1℃, 18～24 時間） BGS 等[3] （36±1℃, 18～24 時間）	1 エーゼ	MLCB 等[4] （36±1℃, 18～24 時間） BGS 等[5] （36±1℃, 18～24 時間）	1 白金耳
確認培養	TSI, LIM（24±2 時間） 培養温度の記載なし		TSI, LIM または LIA 等 （36±1℃, 18～24 時間）		TSI, LIM または LIA 等 （36±1℃, 20～24 時間）	
確認試験	ONPG 試験陽性		血清学的，生化学的試験 （同定キット可）		血清学的，生化学的試験 （同定キット可）	

[1] mBPW: L-システイン 0.2 g/*l* または $FeSO_4 \cdot 7H_2O$ を 64 mg/*l* に添加した BPW
[2] 硫化水素産生により判定する培地で MLCB, DHL, XLD 等
[3] 硫化水素非産生であってもサルモネラと判定できる培地で BGS, BGM（改良 BGA），ランバック培地，SMID 等
[4] 硫化水素産生により判定する培地：MLCB, DHL, XLD, Rainbow Salmonella, ES サルモネラ培地
[5] 硫化水素非産生であってもサルモネラと判定できる培地：BGS, BGM（改良 BGA），ランバック培地，クロモアガーサルモネラ，SMID, ES サルモネラ II
汚染実態調査では，可食部分の外側（キャベツ，レタス，ネギ，タマネギ等），なるべく皮を含む外側（ダイコン，ニンジン，トマト，キュウリ等）を採取

ることである．例えば一次増菌用培地として，殺菌液卵ではL-システインあるいは $FeSO_4 \cdot 7H_2O$ を加えた BPW 培地を，生食用食肉では EEM 培地を使用することになっている．一方，殺菌液卵と汚染実態調査の試験法は基本的には同じであるが，一次増菌用培地 (BPW) へのL-システイン等の添加の有無，RV 培地に接種する一次増菌培地量（0.5 m*l* と 0.1 m*l*）や培養時間の表示が異なる．このようなサルモネラ試験法の現状は，日本の食品細菌試験法の統一性（調和）のなさを象徴しているといっても過言ではない．なお平成 19 年に通知された汚染実態調査の試験法では，一次増菌培地から TT 培地への接種量が 1.0 m*l* に変更された．ついでながら，本通知には大腸菌 (*E. coli*) 試験法も示されている．この中で，「食品，添加物等の規格基準」において大腸菌に係る成分規格が設定されている食品については，当該規格に係る試験検査法を実施すると記載されているが，実際にはそのような成分規格がある食品は存在しない．本記述は不必要であるだけでなく，日本には当該の成分規格があるとの誤解を招きかねない．ちなみに日本の食品の成分規格に採用されているのは E. coli（いわゆる糞便系大腸菌群）である．

7. 腸炎ビブリオ試験法の問題点

平成 13 年の規格基準の一部改正（平成 13 年 6 月 7 日）では，生食用鮮魚介類，生食用冷凍鮮魚介類，むき身にした生食用かき，ゆでがに，ゆでだこに腸炎ビブリオに関する成分規格が設けられた．食肉製品の規格基準改訂時の理念に反して，告示された成分規格に試験法が含まれてしまった（表 8-1）．同時に，告示法と比べて『同等以上の性能を有すると認められる方法により行う』ことも記載された．"同等以上の性能を有する試験法" とは，告示において示された試験法と比較し，特異性および検出感度等において同等または優れている試験法のことであるとされた．この法律が告示されたわずか 22 日後（平成 13 年 6 月 29 日）に，同等以上の性能が認められる試験法が通知された（表 8-2）．

告示法で示された 5% ペプトン含 TCBS 培地は市販されていない（市販培地はすべて 10% ペプトン含）．同等以上の性能が認められる試験法として 10% ペプトン含 TCBS 培地が示されたが，ペプトン以外の組成も微妙に異なるため市販品にはこれに該当するものがない．このため法律を正確に解釈すれば，TCBS 培地は自作しなければならないことになる．同定に使用される TSI 培地，メラーの基礎培地と VP 半流動培地は市販品でもよ

表 8-1. 腸炎ビブリオ試験法（告示法と通知法の比較）

検査項目	告示法			同等以上の性能を有すると認められる試験法（通知法）
	培養		試料の調整	
	温度 (℃)	培地（時間）	試料の希釈倍率と培地の数	
腸炎ビブリオ（定性）	37 37	2%NaCl 加 AP（一夜培養） TCBS（一夜培養） 集落を同定し判定	25 g＋225 m*l* 1 白金耳	○培養温度を 37℃ に代えて，35℃ 以上 37℃ 未満で培養する方法 ○TCBS のペプトン量を 5 g から 10 g に変更した培地，あるいは酵素基質添加培地（クロモアガービブリオ，CHROMagar 社）を使用する方法 ○希釈水にリン酸緩衝生理食塩水[1]を使用する方法 ○希釈別法 10 m*l* (×10)＋2%NaCl 加 AP 90 m*l*—×100 (×100; 10 m*l* そのまま，1 m*l*, 0.1 m*l*/10 m*l* 2% NaCl 加 AP) ×3 本
腸炎ビブリオ (MPN)	37	2%NaCl 加 AP, 10 m*l*（一夜培養）	25 g＋225 m*l* (3%NaCl 加 PB)—×10 ↓1 m*l* 9 m*l* (3%NaCl 加 PB)—×100 (×10; 1 m*l*, ×100; 1 m*l*, 0.1 m*l*)×3 本	
	37	TCBS（一夜培養） 集落を同定し判定	1 白金耳	

[1] リン酸緩衝生理食塩水：PB 原液 1.25 m*l* に 生理食塩水を加えて 1,000 m*l* に調製

表 8-2. 腸炎ビブリオ同定法（通知法）

	温度 (℃)	時 間	判 定
1%NaCl 加 TSI[1]	35～37	18～24	R/Y, H_2S (−), ガス (−)
耐塩性試験[2]	35～37	18	0% (−), 3% (+), 7 又は 8% (+), 10% (−)
1%NaCl 加 VP[1]	35～37	18～24	VP (−)
リシン脱炭酸試験[3]	35～37	1～4日	リシン (+)

[1] 市販の培地に NaCl を最終濃度 1% に加えたものの使用が認められている．
[2] 0, 3, 8, 10%NaCl 加 NB 又は LLB, 又は 0, 3, 7, 8, 10%NaCl 加ペプトン水又はトリプトン水（1% 濃度，pH 7.2）．
NB: Nutrient Broth (Difco, Merck, BBL), LLB: Lab-Lemco Broth (Oxoid)
[3] メラーの基礎培地（Difco，BBL 他）又は LIM 基礎培地に，1%NaCl と 1%L-リシン塩酸基を加えたものを使用．
LIM 基礎培地は市販培地（栄研，日水）と異なる組成が記載されている．
菌接種後，滅菌流動パラフィンを 4～5 mm の厚さに重層して培養．LIM 培地では重層しなくてもよい．

いとなっているが，LIM 培地はそのようになっていない．LIM 培地も TCBS 培地と同様の理由で自作しなければならない．

告示あるいは通知された多くの細菌試験法では，培養温度は例えば 35±1℃，培養時間は 20±2 時間などと表記されている．ところが，告示で示された腸炎ビブリオ試験法では，アルカリペプトン水や TCBS 培地の培養条件は，37℃ で一夜培養となっている．また同等以上の性能が認められる試験法として 35℃ 以上 37℃ 未満で一夜培養する方法も追加された．同定方法で示された TSI 培地と VP 半流動培地の培養時間は 18～24 時間と幅があるが，耐塩性試験では 18 時間となっており，培養温度は 35～37℃ となっている．35～37℃ は 36±1℃ と表記できるが，35℃ 以上 37℃ 未満では 36±1℃ と表記できないし，37℃ という限定された培養温度では恒温槽の性能が考慮されていないため GLP に対応できない．腸炎ビブリオ試験法の記述上の統一性のなさは，地方衛生研究所や登録検査機関等で実施されている GLP システム構築のための SOP 作成に支障をきたすことになる．

最近「同等あるいはそれ以上の性能を有すると認められる試験法」という用語が頻繁に使用されるようになってきた．ところが日本の食品細菌試験法には同等性を評価する方法が示されていない．腸炎ビブリオ試験法が告示された直後に，「同等以上の性能を有すると認められる試験法」が通知されたが，そのデータ等は示されていない．通知法が告示法よりも「同等以上の性能を有すると認められる試験法」であるならば，告示された試験法で成分規格適の食品（腸炎ビブリオが 25 g 中陰性や最確数で 100/g 以下）が，検出感度が優れている試験法では不適となる場合も理論的には起こり得る．化学分析法では，感度が優れた（検出限界値がより低い）試験法を採用すれば検査精度が上昇するのは明らかで，成分規格への適否も数値で容易に判定できる．しかし，細菌試験法では，厳密にいえば試験法の感度は同等でなければ成分規格への適否に影響を与えることになる．

8. 試験法のキャンセルとリプレイスの必要性

サルモネラ試験法の統一性が欠如しているために生ずる弊害の一つとして，2 種類の食品を同時に検査する場合には異なる培地により異なる培養温度等で対応しなければならないことが挙げられる．例えば食肉製品と殺菌

液卵の一次増菌等はそれぞれ 35.0±1.0℃ と 36±1℃（温度表示法が不統一）で培養することになっているので，わずか 1℃ の差であっても，異なる温度設定の培養器が 2 台必要になる．ほとんどの乳製品の試験法の培養温度は 32～35℃ となっているので，乳製品も同時に検査するならば 3 台の培養器が必要になる．このような煩雑さを避けるためにも，新しい試験法を検討する場合には過去の試験法も同時に見直し，必要に応じて ISO のように試験法のキャンセルとリプレイスを明確にすれば検査現場での混乱を防ぐことができると思われる．地方衛生研究所や登録検査機関では告示および通知された試験法に従い SOP を作成し，いわゆる GLP システムで検査を行っている．この現状を十分に留意して，統一性（調和）のある試験法を作成することが必要であると考えられる．より調和のとれた試験法は，GLP に対してよりシンプルに対応が可能であり，結果として，単純な検査ミスをなくすることにも寄与するものと思われる．

最終的には，今までに告示あるいは通知された試験法を個々に見直すのではなく，抜本的かつ系統的に改正することによってのみ，調和のとれた試験検査法が構築できるのではないかと考える．

9. 最 後 に

本稿では食品の細菌試験法の現状とその現実的な問題点に焦点を絞ったが，根本的には成分規格そのものの妥当性を議論することが重要である．日本の食品衛生法では，腸管系食中毒菌に対する汚染指標菌として大腸菌群と E. coli（糞便系大腸菌群）が使用されている．ところが，2006 年から施行されたヨーロッパ連合の食品に対する微生物基準から大腸菌群と糞便系大腸菌群がなくなり，大腸菌と *Enterobacteriaceae*（仮訳：腸内細菌科菌群）に置き換えられた．これらの指標菌は最終製品（市販食品）ではなく，HACCP に対応可能な製造工程での衛生管理基準に使用されており，市販食品の安全基準には食中毒菌そのものが使用されている．米国でも糞便系大腸菌群の検査頻度は低下しているようである．また，食品の良否を判定する方法として，日本のような単品の抜き取り検査ではなく，ICMSF（国際食品微生物規格委員会）のサンプリングプランに従ったロット検査が実施されている．

このような食品の試験検査法の国際動向を視野に入れながら，日本の食品衛生のあるべき姿を追い求めることは，日本食品微生物学会の重要な課題の一つであると考える．

Original

Quantitative Duplex PCR of *Clostridium botulinum* Types A and B Neurotoxin Genes

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A duplex quantitative polymerase chain reaction (PCR) assay for *Clostridium botulinum* types A and B was developed. The sensitivity and specificity of the assay were verified by using 6 strains of type A, 7 strains of type B, and 14 genera of 42 non-*C. botulinum* types A and B strains, including *C. botulinum* types C, D, E, F, and G. In pure culture, the detection limit was 10^2 CFU/mL for type A and 10^3 CFU/mL for type B. In mushroom broth, increases in the amounts of *C. botulinum* types A and B could be monitored separately (the quantifiable range was 10^2 to 10^6 for type A and 10^2 to 10^7 for type B) from each sample that contained a large number of background bacteria, and toxin could be detected much earlier than with mouse assay. These results suggest that duplex quantitative PCR methods are useful to detect and quantify *C. botulinum* types A and/or B toxin genes.

Key words: *Clostridium botulinum*; quantitative PCR; duplex; TaqMan

Introduction

Clostridium botulinum is a spore-forming obligative anaerobic bacterium that is well known to produce the most potent neurotoxins, designated A to G[1]. Types A, B, and E usually cause human botulism[2]. The main human pathogens are found in group I, which consists of proteolytic types A, B, and F, and in group II, which consists of nonproteolytic types B, E, and F. These two groups are completely different in their physiological aspects, not only regarding proteolysis, but also fermentation of sugars, metabolic acids, growth temperature, and heat resistance[3,4].

Human foodborne botulism of types A and B generally occurs in temperate zones in the western United States, Argentina, Brazil, and China for type A and in Poland, Czechoslovakia, Hungary, Yugoslavia, Germany, Belgium, France, Italy, Spain, and Portugal for type B[5]. Occasionally, both types have been implicated in the same incident[5]. Some types can produce both kinds of toxins. Franciosa reported that 42 of 79 strains of *C. botulinum* type A isolated in the United States were encoding both *botA* and *botB*[6,7].

Detection of *C. botulinum* cells or their neurotoxins in foods is the first step in risk analysis or risk assessment of foodborne botulism. Currently, mouse bioassay is still the major detection method for *C. botulinum* because of its sensitivity and reliability. In spite of these advantages, the assay is costly, time-consuming, laborious, and requires handling of laboratory animals. Thus, only a limited number of samples can be analyzed at one time using the mouse bioassay. To improve this situation, some rapid alternative detecting methods have been developed. Currently, together with the mouse assay, the enzyme-linked immunosorbent assay (ELISA) is most widely used for food analysis[8]. However, ELISA has several deficiencies, including sensitivity, complexity in handling, and accuracy. However, an ELISA that is sensitive for type B has been described[9] as being more sensitive than the mouse bioassay, and seems to have overcome these deficiencies. Immuno-PCR is another rapid and sensitive detecting system[10,11] in which a reporter DNA molecule is used instead of an enzyme conjugated to an antibody. The sensitivity of immuno-PCR for detecting type A neurotoxin is 10^3-fold to 10^5-fold higher than that of traditional ELISA[10,11]. Multiple alternative methods should be developed and evaluated for performance, cost, and suitability for automation in the food industry.

Amplification by PCR has become an important method for rapidly, sensitively and specifically assaying a target gene. Many authors have reported the detection of *C. botulinum* in foods not only by simplex PCR, but also by multiplex PCR[12-22]. However, all of these studies required electrophoresis. Electrophoresis is time-consuming, has a risk of cross-contamination when caps are reopened, requires the handling of carcinogenic chemicals to stain the gel, and must be judged visually from the gel image. The approach to risk analysis or risk assessment of botulism should be simpler and the data should be more easily obtainable

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for prompt access.

Recently, real-time PCR has become widely used for the detection and/or quantification of bacterial genes. Some researchers have reported the detection and/or quantification of *C. botulinum* neurotoxin genes for type A[23], for types A, B, and E[24, 25], for types A and B[26], and for type E[27]. Other real-time PCR approaches, which involve quantification of RNA expression, were reported for types A and E[28], for type B[29, 30], and for type D[31]. These representative studies did not use duplex PCR quantification methods.

In this paper, we describe the detection and quantification of *C. botulinum* types A and B by means of a quantitative duplex PCR method. We also describe the estimation of *C. botulinum* types A and B in mushroom broth with both simplex and duplex PCR methods.

Materials and Methods

Bacterial strains and culture conditions

The clostridium and non-clostridium strains tested in this study are listed in Table 1. The clostridium strains were cultured anaerobically in GAM (Gifu anaerobic medium, Eiken Chemical Co., Tokyo, Japan) broth in an anaerobic chamber (Hirayama type J or BBL Gaspak system) equipped with a deoxidizer (Anaeropack Anaero, Mitsubishi Gas Chemical Co., Tokyo, Japan). Non-clostridium strains were cultured aerobically in TSB (trypticase soy broth, Difco, Sparks, MD, USA). All cultures were incubated at 30℃ or 37℃.

C. botulinum types A and B spore preparation

The *C. botulinum* types A and B strains were precultured anaerobically with cooked meat medium or GAM broth at 37℃ overnight. Spores of each strains were produced in a modified Gibbs's TPY (trypticase peptone yeast extract)[32] broth (pH 7.0), consisting of 5.0% trypticase peptone (Difco), 0.5% bactopeptone (Difco), and 0.1% bacto yeast extract (Difco), at 37℃ over 7 days. Spores were checked for the dominance of refractile spores (>90%) with a phase-contrast microscope (BX50, Olympus Co., Tokyo, Japan) before harvesting. Spore crops of each strain were centrifuged at 21,480×*g* for 10 min at 4℃ (high-speed refrigerated centrifuge SRX-201, Tomy Seiko Co., Tokyo, Japan). The pellets were washed five times with ice-cooled sterile distilled water. Each crop was resuspended in sterile distilled water and stored at −20℃ or 5℃ before use. Spores of each strain were counted in clostridia count agar (Nissui Pharmaceutical Co., Tokyo, Japan) medium by the pouch method[27], after heat-shock treatment (80℃ for 10 min, then rapid cooling). Primers and probes were designed for *C. botulinum bot*A (X52066) and *botB* (M81186) from the DDBJ (DNA Data Bank of Japan) database (DDBJ, 2004).

Primers and probes

Selection of primers and probes allowed for adjustment of the melting temperatures to those optimal for the ABI 7700 sequence detection system (Applied Biosystems division of Perkin-Elmer Co., Foster City, CA, USA). A 180-bp gene for type A and a 195-bp gene for type B were selected for specific amplification after the nucleotide sequences of *botA*, *botB*, *botC*, *botD*, *botE*, *botF*, *botG*, and *tet* (X52066, M81186, D49440, D38442, AB082519, M92906, X74162, and X06214, respectively) were aligned using Clustal W software, version 1.74[33]. The sequences for type A and type B primers are listed in Table 2.

Quantitative PCR for types A and B

Each 50-μL PCR reaction mix contained: 1X TaqMan buffer A; 200 mM each dATP, dCTP, and dGTP; 400 mM dUTP; 200 nM primers (Funakoshi Co., Ltd., Tokyo, Japan), and 100 nM probe (Qiagen, K. K.) for type A or 100 nM primers (Funakoshi) and 100 nM probe (Qiagen) for type B; 5.0 mM $MgCl_2$; 0.5 U of uracil-*N*-glycosidase (Amp Erase UNG; Applied Biosystems); 1.25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems); and 5 μL as a template. The reporter fluorescence dye FAM was used for both types A and B and the quencher used was TAMRA.

Reactions were run on an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) under the following conditions: 2 min at 50℃, 10 min at 95℃ and 50 cycles of 15 sec at 95℃, and 60 sec at 63℃ for both types A and B.

Quantitative duplex PCR

Each 50-μL duplex PCR reaction mix contained: 1X TaqMan buffer A; 200 mM each dATP, dCTP, and dGTP; 400 mM dUTP; 100 nM primers (Funakoshi) and 100 nM probe (Qiagen) for type A, 400 nM primers (Funakoshi) and 200 nM probe (Qiagen) for type B; and 5 μL of template. The reporter fluorescence dye used for type A probe was FAM and that for type B was VIC. TAMRA was used as a quencher dye for both types A and B.

Reactions were run on the same equipment using the same quantitative PCR conditions for both types A and B.

Standard curve and PCR efficiency

The standard curve was constructed following Kimura's method[27]. All PCR efficiency measurements were made in triplicate.

Detection limit from pure culture

The detection limit was calculated with 56A and Okra from pure culture by both simplex and duplex PCR methods. The genomic DNA was extracted by the guanidine isothiocyanate method[27, 34]. The detection limit was also tested in triplicate.

Growth curve of C. botulinum in pure culture

Dilutions of overnight culture (final concentration was 8.0 CFU/mL) of *C. botulinum* type A (56A) or type B (QC: group II) were inoculated into Erlenmeyer flasks containing 800 mL of GAM broth and incubated at 37℃

Table 1. Strains tested by the quantitative PCR method

Species	Type	Strain	Source[a]	PCR	
				Tpe A	Type B
Clostridium botulinum	A	56A	SCHU	−	−
		62A	SCNIH	+	−
		97A	SCNIH	+	−
		Hall	SCHU	−	−
		Kyoto F	SCHU	+	−
	A (silent B)	Renkon-1	SCHU	−	−
	B	9B	SCNIH	−	+
		213B	SCHU	−	+
		407-1	SCHU	−	+
		Fukuyama	SCHU	−	+
		Okra	SCNIH	−	+
		Karashi	SCHU	−	+
		QC	SCHU	−	+
	C	003-9	SCHU	−	−
	D	Karugamo	SCHU	−	−
	E	164-1	SCHU	−	−
		5545	SCHU	−	−
		Iwanai	SCHU	−	−
		Tenno2	SCHU	−	−
		35296	SCHU	−	−
		Biwako	SCHU	−	−
	F	4257	SCHU	−	−
		9H-01F	SCHU	−	−
		Cardella	SCHU	−	−
		Langeland	SCHU	−	−
		Yaeyama	SCHU	−	−
	G	G2734	SCHU	−	−
		G2741	SCHU	−	−
Clostrdium butylicum		1443	SCHU	−	−
Clostridium perfringens		BBC 2401	BBC	−	−
Clostridium sporogenes		IFO 13950	IFO	−	−
Clostridium subterminale			Meat	−	−
Bacillus cereus		IFO13494	IFO	−	−
Bacillus coagulans		IFO12583	IFO	−	−
Bacillus licheniformis		IFO12200	IFO	−	−
Bacillus subtilis		IFO13719	IFO	−	−
Campylobacter coli		ATCC36887	ATCC	−	−
Campylobacter jejuni		ATCC33560	ATCC	−	−
Escherichia coli		ATCC11775	ATCC	−	−
Listeria monocytogenes		ATCC7644	ATCC	−	−
				−	−
				−	−
Listeria innocua		ATCC33070	ATCC	−	−
Morganella morganii		ATCC25830	ATCC	−	−
		JCM1672	JCM	−	−
Pseudomonas fluorescens		IFO14160	IFO	−	−
Raoultella planticola		ATCC43176	ATCC	−	−
Salmonella enterica serovar Typhimurium		IFO13245	IFO	−	−
Serratia marcescens			Fish	−	−
Staphylococcus aureus		ATCC12600	ATCC	−	−
Tetragenococcus muriaticus		JCM10006	JCM	−	−
Vibrio parahaemolyticus		IFO 12711	IFO	−	−
Yersinia enterocolitica		ATCC9610	ATCC	−	−

[a] SCHIH: G. Sakaguchi's Collection at National Institute of Health, Japan (given by S. Igimi); SCHU: G. Sakaguchi's Collection at Hiroshima University, Hirosihma, Japan; ATCC: American Type Culture Collection, T. Manassas, Va.; BBC: Japanese Association of Veterinary Biologics; JCM: Japan Collection of Microorganisms, Saitama, Japan; IFO: Institute for Fermentation, Osaka, Japan.

Table 2. Primer and fluorogenic probe sequences for *botA* and *botB* detection

Name	Terget gene	Sequence	
CbA 591-617F	*botA*	5′-GGAGTCACTTGAAGTTGATACAAATCC-3′	This study
CbA 770-744R	*botA*	5′-TCTAACCCACTCATTTCATAATAGGCA-3′	This study
CbA 669-727	*botA*	5′-F-TAGGTGCAGGCAAATTTGCTACAGATCCA-Q-3′	This study
boNB400	*botB*	5′-AGACGTGTTCCACTCGAAGAGTTT-3′	This Study
boNB600R	*botB*	5′-GCCTTCCCTTGATGCAAAATG-3′	Christensen *et al.* [12]
BP-4050	*botB*	5′-F-TCAGTAATCCAGGAGAAGTGGAGCGAA-Q-3′	This study
BP-4050V	*botB*	5′-V-TCAGTAATCCAGGAGAAGTGGAGCGAA-Q-3′	This study

F: reporter dye (FAM), Q: quencher dye (TAMRA), V: reporter dye (VIC).

for type A and at 30℃ for type B for 24 hr. The number of *C. botulinum* cells during growth was determined by Kimura's method[27].

Growth in GAM broth with mushrooms

Mushrooms (*Agaricus bisporus*) purchased at a local retail store were cut in half (total 80 g). One half was incubated in 720 mL of GAM broth with eight strains [four strains of type A (56A, 62A, 97A, and Hall) and four strains of type B (9B, 213B, 407-1, and Okra)] of *C. botulinum* spores and the other was incubated in 720 mL of GAM broth without inoculation. The inoculum contained 8×10^4 CFU of each strain. The inoculum was heatshocked at 80℃ for 10 min and cooled rapidly in ice water. After the heatshock treatment, 1 mL of the mixture was inoculated into 800 mL of GAM broth with mushrooms. The final concentration of *C. botulinum* was about 1×10^2 CFU/mL. The cultures were incubated anaerobically at 37℃ in an anaerobic chamber equipped with a deoxidizer. The sampling was carried out every 2 hours from time 0 to 24 hr. Viable anaerobic cell count was determined at every sampling time by serial dilution and pour method using GAM agar, and about 13 mL of culture was also stored at −20℃ every sampling time for the TaqMan PCR and mouse assays.

Neurotoxin assay

Frozen cultures were thawed and mixed sufficiently. The culture samples were centrifuged at $2,190\times g$ for 10 min. Toxicity was assayed by intraperitoneally injecting the supernatant (0.5 mL) into each of two mice (body weight: about 20 g). Samples were judged to be toxic if both mice died over the 48-hr observation period[35]. The neurotoxin detected in samples was tested for serum type by inoculation into A and/or B antiserum-protected mice (2 mice for each treatment; total, 6 mice). Antiserum protection was established with an intraperitoneal injection of 0.5 mL of a mixture of 2 U/mL adjusted types A and/or B antiserum and the same volume of sample incubated for 1 hr at room temperature. If the A antiserum-protected mice died and the B antiserum-protected mice lived during the 48-hr observation, the serum type was judged to be B. If only B antiserum-protected mice died and the A antiserum-protected mice lived, the serum type was considered to be A. If only A and B double-protected mice lived and the others died, the serum type was judged to be both A and B.

Results

Specificity of primers and probes for C. botulinum types A and B

The specificity of primers and probes is shown in Table 1. All six type A strains (56A, 62A, 97A, Hall, Kyoto F, and Renkon-1) were positive with the type A primers and probe set, and all seven type B strains (9B, 213B, 407-1, Fukuyama, Okra, Karashi, and QC) were positive with the type B primers and probe set. Only the Renkon-1 strain (A, silent B: type B toxin genes are coded for, but are nonfunctional) was positive with both type A and type B primers and probe sets, while other positive strains were distinguishable by *only one* type-specific primer and probe set. These samples gave C_t values of <25. All of the 14 genera of 42 non-*C. botulinum* types A and B strains, including *C. botulinum* types C, D, E, F, and G, were nonreactive to the probes with Ct values of >50 cycles, confirming the species-specific nature of the assay (Table 1).

PCR amplification efficiency

Amplification efficiency of each *C. botulinum* strain was calculated from each standard curve with six strains of type A (56A, 62A, 97A, Hall, Renkon-1 with silent B, and the infant botulism strain Kyoto F) and six strains of type B (9B, 407-1, Fukuyama, Okra, nonproteolytic Karashi, and nonproteolytic QC) (Table 3). Both the type A (56A) and type B (QC) PCR amplification plots are shown in Fig. 1. The silent B strain was also checked for amplification efficiency of the *botB* gene (Table 3). The amplification efficiency using duplex PCR was also calculated for representative strains (56A, Renkon-1, and Okra) (Table 3).

Detection limit from pure culture

The detection limit of *C. botulinum* 56A (type A) was found to be 2.8×10^1 CFU/mL by both simplex PCR and duplex PCR. That of Okra (type B) was found to be 4.7×10^1 CFU/mL by simplex PCR and 4.7×10^2 CFU/mL by duplex PCR.

Evaluation of cell growth by the quantitative PCR method and pouch method in pure culture

Evaluation of cell growth by the quantitative PCR

Table 3. PCR amplification efficiency

Organism	Type	Primers and probes	Strain	Reporter	Efficiency	Detection limit [fg]
Clostridium botulinum	A	A	56A	FAM	0.92	50
	A	A	62A	FAM	0.97	50
	A	A	97A	FAM	0.94	50
	A	A	Hall	FAM	0.97	50
	A	A	Kyoto F	FAM	0.99	50
	A (silent B)	A	Renkon-1	FAM	0.93	50
	B	B	9B	FAM	0.94	50
	B	B	407-1	FAM	0.94	50
	B	B	Fukuyama	FAM	0.94	50
	B	B	Okra	FAM	0.94	50
	B	B	Karashi (non proteolytic)	FAM	0.95	50
	B	B	QC (non proteolytic)	FAM	0.94	50
	A (silent B)	B	Renkon-1	FAM	0.91	50
	A	A and B	56A	FAM	0.88	50
	A (silent B)	A and B	Renkon-1	FAM	0.83	50
	A (silent B)	A and B	Renkon-1	VIC	0.87	50
	B	A and B	Okra	VIC	0.81	500

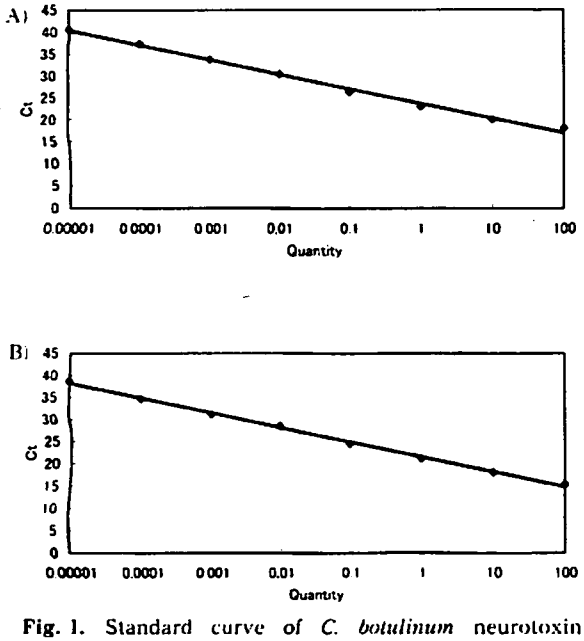

Fig. 1. Standard curve of *C. botulinum* neurotoxin genes by simplex PCR

A) Strain 56A (*botA*), B) Strain QC (*botB*).

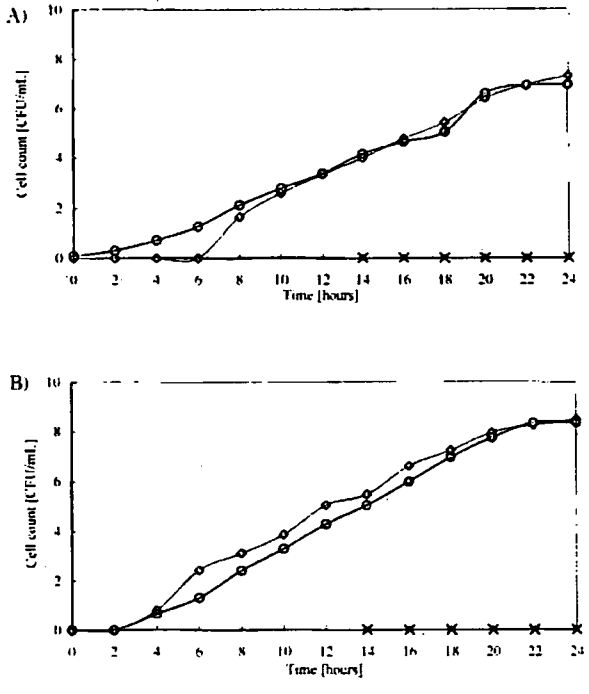

Fig. 2. Growth curve of *C. botulinum* in GAM broth with a pure culture. A) Strain 56A (type A) incubated at 37°C. B) Strain QC (type B) incubated at 30°C.

◇, quantified by quantitative PCR; ○, quantified by culture method. Solid symbols denote the samples detected with neurotoxin by mouse assay. X, carrying mouse assay.

method and by the conventional pouch method was made. Similar growth curves were obtained with both the quantitative PCR and culture methods for both type A (56A) and type B (QC) cultures (Fig. 2). Neurotoxin was detected by mouse assay after 16-hr incubation of both types and the viable cell count at that time was about 10^5 CFU/mL for type A (56A) and 10^6 CFU/mL for type B (QC) (Fig. 2). These results indicate that quantitative PCR could be used instead of the conventional culture method for monitoring *C. botulinum* growth. Also, it was clear that the quantitative PCR method was more sensitive than the mouse assay.

Botulinal growth in GAM with mushroom broth

When eight strains of *C. botulinum* (type A: 56A, 62A, 97A, and Hall; type B: 9B, 213B, 407-1, and Okra) were inoculated into mushroom broth, *C. botulinum* was detected and type A and B genes could be distinguished at the same time from all extracted samples by the quantitative duplex PCR method, while no signals were

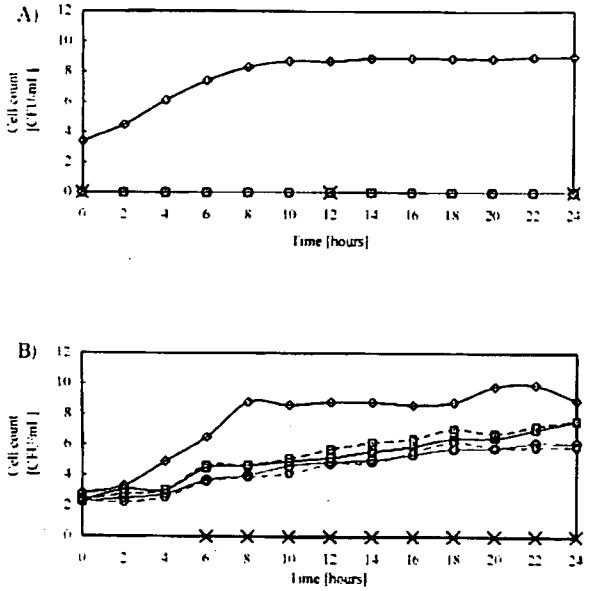

Fig. 3. Cell count of *C. botulinum* in mushroom broth

No inoculation (A). Inoculation of *C. botulinum* types A (56A, 62A, 97A, and Hall) and B (9B, 213B, 407-1, and Okra) (B).
◇, anaerobic count by GAM agar; ◇, estimation of cell number of type A by quantitative simplex PCR (dashed line) and by quantitative duplex PCR (solid line); □, estimation of cell number of type B by quantitative simplex PCR (dashed line) and by quantitative duplex PCR (solid line); X, mouse assay. Solid symbols denote the samples in which neurotoxin was detected with mouse assay.

obtained from uninoculated samples throughout the experiment (Fig. 3). The background anaerobic cell count checked by GAM agar was from 4.0×10^{0} to 1.6×10^{9} CFU/mL in inoculated broth and from 2.5×10^{3} to 1.0×10^{9} CFU/mL in uninoculated broth. The toxin was detected from only inoculated samples by mouse assay. The first detection of the toxin was 14 h after the inoculation as type B toxin. After 16-hr incubation, type A toxin was also detected (Fig. 3). It was clear that the quantitative duplex PCR method was also more sensitive than the mouse assay.

Discussion

Detection of *C. botulinum* neurotoxin genes by conventional PCR and agarose gel electrophoresis has been reported by many researchers, using not only simplex PCR, but also multiplex PCR[6,7,12-17,19-22,36-40]. Real-time PCR detection of neurotoxin genes was also reported[23-27]. Among these studies, quantitation for type A was reported by Yoon *et al.*[23] and quantitation for type E was described by Kimura *et al.*[27] Real-time quantitative PCR has a number of advantages as compared to conventional assays for the detection or quantification of foodborne bacteria. The results are shown as fluorescence intensity, and the absence of electrophoresis eliminated the need for reopening of the reaction tubes, thus limiting the risk of carry-over contamination.

We developed quantitative simplex PCR for *C. botulinum* type A/B and quantitative duplex PCR for *C. botulinum* types A and B. The order of the detection limit was 10^{1} CFU/mL by both simplex and duplex PCR for type A and simplex PCR for type B, and 10^{2} CFU/mL by duplex PCR for type B. The sensitivity of the simplex PCR is the same as that of duplex PCR for type A, but for type B, it was almost 10 times higher than that of duplex PCR in our results. This suggests that the type A primers and probe set may inhibit amplification of the target region of the type B set.

Actually, quantifying neurotoxin DNA is not quantifying the neurotoxin itself. Some researchers have evaluated the mRNA expression of the neurotoxin gene by competitive reverse transcription (RT)-PCR[41-43] and real time RT-PCR[28-31].

Quantifying mRNA is more appropriate for risk assessment of *C. botulinum* compared with quantifying neurotoxin DNA[27]. However, the handling of DNA is simpler than the handling of mRNA. It is also known that *C. botulinum* neurotoxin is generally detected in the late exponential phase in pure culture[11]. An increase in neurotoxin mRNA reflects a potential risk of neurotoxin production. Detection and quantitation of neurotoxins have been performed by ELISA assay[8,9,45-48], immuno-PCR assay[10,11], and liposome-PCR (LPCR) assay[49]. The ELISA assay has several deficiencies, including sensitivity, complexity in handling, and accuracy. The sensitivity of immuno-PCR for detecting type A neurotoxin is reportedly 10^{3}-fold to 10^{5}-fold higher than that of traditional ELISA[10,11]. The most sensitive method is LPCR, for which the detection threshold is 0.02 fg/mL compared with that of the mouse bioassay of 9 pg/mL[49]. However, those highly sensitive methods were performed *in vitro*, not with foods or environmental samples, which would contain bacteria themselves, bacterial metabolites, and debris from foods or the environment. It is still unclear how useful these methods are for evaluating foods or environmental samples.

Mushroom is a secondary decomposer, which means that bacteria and other fungi have to break down raw materials before *Agaricus* can grow, so the mushroom needs to grow in compost. Further, production of the fruiting body requires *Pseudomonas putida*[50]. So, the mushroom would contain *Pseudomonas* spp. and other bacteria, such as heat-resistant spore formers, derived from the raw materials of compost (maturing compost can reach a temperature of up to 80°C). Actually, *C. botulinum* has been identified in mushroom[51,52]. Fresh mushrooms consume oxygen and produce carbon dioxide through respiration when they are packaged with plastic film. In these circumstances, *C. botulinum* can grow and form toxin [52]. With both our simplex and duplex PCR methods, the growth of *C. botulinum* types A and B could be detected and quantified in mushroom broth containing a large number of background bacte-

ria derived from the mushroom. Dezfulian reported that *Clostridium botulinum* isolation (CBI) medium is useful for detecting *C. botulinum*[53]. However, the viable cell count on CBI medium was at least 100 times lower than that on Clostridia medium in pure culture (data not shown), because CBI medium is a selective medium and Clostridia medium is not. It is, therefore, useless to estimate the viable cell count of *C. botulinum* with CBI medium. Our method would be useful and effective for estimating the viable cell count of *C. botulinum* types A and B in samples that contain large numbers of background bacteria.

In conclusion, the quantitative duplex PCR method could be a significant development for the quantification of *C. botulinum* types A and B toxin genes from pure culture and mushroom broth. The efficiency of the quantitative simplex PCR method was higher than that of the duplex method, but a good correlation was found between the results of the simplex and duplex PCR methods in mushroom broth. This suggests that the quantitative duplex PCR method could be useful for determining the numbers of *C. botulinum* types A and B in foods.

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Nonsense-mutated *inlA* and *prfA* not widely distributed in *Listeria monocytogenes* isolates from ready-to-eat seafood products in Japan

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Abstract

InlA is a surface protein participating in the entry of *Listeria monocytogenes* into mammalian non-phagocytic cells. PrfA is a positive regulatory factor that regulates the expression of a set of virulence genes. Recent studies revealed that some *L. monocytogenes* strains have a truncated form of these proteins because of nonsense mutations in their sequences, and these truncations contribute to the significant reduction in virulence of this pathogen. In this study, sequence analyses of *inlA* and *prfA* among *L. monocytogenes* isolated from ready-to-eat seafood revealed that only one out of 59 isolates had a nonsense-mutated *inlA* and all had non-mutated *prfA*. This indicated that these strains could be fully virulent based on the sizes of these proteins.

Keywords: *Listeria monocytogenes*; Internalin A; Ready-to-eat; Seafood

1. Introduction

Listeria monocytogenes is an ubiquitous bacterium that can cause serious listeriosis infections in humans and animals. Both sporadic and epidemic cases of human listeriosis are mainly of food-borne in origin and have an associated mortality rate as high as 20–30% (Mead et al., 1999). Healthy adults are generally asymptomatic or develop only mild symptoms with simple gastroenteritis (Grif et al., 2001; Rocourt et al., 2000). However, infection in high-risk individuals, such as pregnant women, newborn infants, and immunocompromised people, can result in serious outcomes such as spontaneous abortion, septicemia, and meningoencephalitis. *L. monocytogenes* is therefore a public concern in terms of food safety and regulations to control this organism have been established in many countries. However, acceptable levels of this organism in ready-to-eat foods are defined differently from country to country. The United States adopted a zero-tolerance policy for all ready-to-eat foods whereas the EU allows 100 CFU/g of this pathogen at the best-before date for some classes of foods (European Commission, 2005). Establishing a definitive universal policy on acceptable levels of this organism is definitely required, and to this end, risk analysis is necessary to understand the actual dose response. However, it should be noted that these policies have been established based on the hypothesis that all *L. monocytogenes* strains are equally pathogenic, despite the heterogeneity of pathogenicity that has been reported to exist among isolates. This is indicated by most of the human listeriosis cases having been caused by strains of certain serotypes, such as 1/2a, 1/2b and 4b (Schuchat et al., 1991). Specifically, the strains of serotype 4b have been responsible for most food-borne epidemic listeriosis cases and the majority of sporadic cases (Farber and Peterkin, 1991; Schuchat et al., 1991). The varying levels of virulence were also demonstrated by virulence tests using chick embryo, various human cell lines, and mouse injection test (Bhunia et al., 1994; Nørrung and Andersen, 2000; Pine et al., 1991; Roberts et al., 2005; Roche et al., 2003; Roche et al., 2001; Stelma et al., 1987; Tabouret et al., 1991; Van Langendonck et al., 1998).

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The factors for this heterogeneity in virulence have been elucidated by various molecular methods. The observation that some *L. monocytogenes* isolates express a truncated non-functional form of internalin A (InlA) is of particular importance (Jonquières et al., 1998). InlA allows the pathogen to invade non-phagocytic cells, such as human intestinal epithelium cells (Gaillard et al., 1991), but strains expressing truncated InlA show a significant reduction in invasive ability into Caco-2 cells compared to ones lacking the nonsense mutation (Olier et al., 2005; Olier et al., 2002; Rousseaux et al., 2004). A truncated form of InlA was shown to be widely distributed in food isolates and less so in clinical isolates (Jacquet et al., 2004), indicating the critical role of InlA in the pathogenesis of human listeriosis. Reasons for strains of serotype 4b posing high risks for humans is still unknown, but having the intact form of *inlA* gene, rather than a nonsense-mutated form found in other strains, may play a partial role (Jacquet et al., 2004). In another recent study, truncation of this protein in a number of clinical and food isolates was confirmed using isolates from the United States (Nightingale et al., 2005a). Moreover, nonsense mutations were also found in the *prfA* gene (Roche et al., 2005), which regulates expression of a set of virulence factors. Although only three isolates were found to have nonsense-mutated *prfA*, all of them failed to enter human adenocarcinoma cells and were either avirulent or hypovirulent to mice because of their truncated PrfA proteins (Roche et al., 2005).

This virulence attenuation mechanism sheds light on questions about the rate of listeriosis cases. Although *L. monocytogenes* is widely present in ready-to-eat foods (Gombas et al., 2003), the number of cases of human infection is relatively low. This is also true in Japan where almost no foodborne listeriosis cases have been reported to date, although *L. monocytogenes* is known to be prevalent in many kinds of foods (Okutani et al., 2004). Despite significant consumption of these foods in Japan, it should be particularly noted that raw fish and ready-to-eat raw fish products have never been implicated in listeriosis in humans. This may be due to the low cell number of *L. monocytogenes* in these foods. Or, the presence of *L. innocua*, which is commonly found in foods (Karunasagar and Karunasagar, 2000), enhances host protective immunity against this pathogen (Vázquez-Boland et al., 2001). Alternatively, previous outbreaks simply have escaped recognition since *Listeria* detection from patients with diarrhea has not been routinely performed (Makino et al., 2005). However, the possibility that non-virulent or virulence-attenuated strains are prevalent in these foods cannot be ignored. Therefore, it is of extreme importance to determine whether truncation of virulence or virulence-associated genes could be a new tool for assessing risk of consuming food products contaminated with *L. monocytogenes*. Thus, we investigated *L. monocytogenes* isolates in ready-to-eat seafoods in this study to determine whether virulence-related genes *inlA* and *prfA* have nonsense mutations that leads to the truncated form of their respective proteins, InlA and PrfA. As sample foods, we specifically selected fish roe and minced tuna, since these have high levels of *L. monocytogenes* contamination (Handa et al., 2005) and risk assessment of these foods is urgently required.

2. Materials and methods

2.1. Bacterial isolates

The 59 seafood isolates used in this study are summarized in Table 1. A total of 10 isolates were from a previous study (Handa et al., 2005) and an additional 49 were selected from 64 isolates obtained from 531 ready-to-eat raw seafood retail products obtained in 61 different grocery stores in and around Tokyo between October 2004 and July 2005. The remaining15 isolates were excluded from further analyses because isolation sources (sampling date and store number), EcoRI ribotyping (Bruce, 1996) or MLST (Maiden et al., 1998; Zhang et al., 2004) data suggested that they were clonal isolates of other isolates already included in our list. Strains of the same serotype, ribotype and MLST profile were included in this study when the food samples were obtained on different dates or different stores.

2.2. Serotyping

Serotyping was carried out with commercial *Listeria* antiserum (Denka Seiken, Tokyo, Japan). O-antigen determination strains were grown on brain heart infusion agar (Becton Dickinson, Sparks, MD, USA) for 24 h at 35 °C. Cells were suspended in 0.2% sodium chloride and heated at 121 °C for 30 min followed by centrifugation at 3000 rpm for 20 min and resuspended in 0.5 ml of 0.2% sodium chloride. Slide agglutination tests using polyvalent type O-antiserum were performed first, followed by typing with individual O-antiserum. H-antigen strains were determined using the tube agglutination test. Briefly, sample cultures were incubated in semiliquid BHI medium (0.2% wt/vol agar) at room temperature (20–25 °C) for 24 h, repeated four times. The samples were incubated in a semiliquid BHI medium in Craigie tubes for 24 h followed by removal to BHI medium for an additional 24-h incubation. H-antigen type was determined after mixing two drops of antiserum with 0.5 ml of cell suspension with 1% formalin and incubating at 50 °C for 1 h.

2.3. Lineage designation

L. monocytogenes has been grouped into 3 distinct phylogenetic lineages based on genotypings such as sequencing analysis, ribotyping, and PCR-restriction fragment length polymorphisms (Rasmussen et al., 1995; Wiedmann et al., 1997). Each of the 59 strains used in this study was categorized into one of these 3 lineages using a method described previously (Ward et al., 2004). This method used multiplex PCR to produce a lineage-specific sized band on electrophoresis gels.

2.4. MLST (multilocus sequence typing)

Partial regions of 6 different virulence and virulence-associated genes were selected for MLST analysis according to Zhang et al. (2004) since they have reported a high discriminatory power of this method. DNA sequencing for each locus was performed

Table 1
L. monocytogenes strains isolated from ready-to-eat raw seafood

Strain	Serotype	Lineage	MLST type	Ribotype	Sampling date	Store no.	Sample type	Nonsense mutation		Reference
								inlA	*prfA*	
2-9	1/2a	II	18	1056	19-Nov-02	2	Salmon roe "sujiko"	–	–	Handa et al. (2005)
5-2	1/2a	II	14	1023	10-Dec-02	2	Cod roe "tarako"	–	–	Handa et al. (2005)
5-4	1/2a	II	26	1046	10-Dec-02	2	Salmon roe "sujiko"	–	–	Handa et al. (2005)
6-9	1/2a	II	12	1023	15-Dec-02	3	Minced tuna	–	–	Handa et al. (2005)
11-4	1/2a	II	20	1030	15-Jun-03	5	Cod roe "tarako"	–	–	Handa et al. (2005)
12-17	1/2a	II	12	1030	22-Jun-03	6	Minced tuna	–	–	Handa et al. (2005)
12-18	1/2a	II	9	1027	22-Jun-03	7	Cod roe "tarako"	–	–	Handa et al. (2005)
13-20	1/2a	II	11	1039	5-Nov-03	8	Minced tuna	–	–	Handa et al. (2005)
20-7-1	1/2a	II	15	16619	28-Oct-04	9	Cod roe "mentaiko"	–	–	This study
22-13-3	1/2a	II	13	1023	16-Nov-04	10	Minced tuna	–	–	This study
22-18-5	1/2a	II	20	1035	16-Nov-04	11	Minced tuna	–	–	This study
22-29-1	1/2a	II	24	1053	16-Nov-04	12	Cod roe "tarako"	–	–	This study
23-4-4	1/2a	II	12	16619	25-Nov-04	13	Salmon roe "sujiko"	–	–	This study
23-29-1	1/2a	II	9	1030	25-Nov-04	14	Salmon roe "sujiko"	–	–	This study
25-4-1	1/2a	II	12	16619	9-Dec-04	15	Salmon roe "sujiko"	–	–	This study
25-8-1	1/2a	II	22	1035	9-Dec-04	11	Minced tuna	–	–	This study
25-15-1	1/2a	II	25	1045	9-Dec-04	10	Cod roe "mentaiko"	–	–	This study
26-1-2	1/2a	II	12	1030	13-Jan-05	10	Minced tuna	–	–	This study
26-26-2	1/2a	II	8	1030	13-Jan-05	14	Salmon roe "sujiko"	–	–	This study
28-9-1	1/2a	II	16	1039	3-Feb-05	16	Salmon roe "ikura"	–	–	This study
29-13-2	1/2a	II	12	16619	17-Feb-05	18	Cod roe "mentaiko"	–	–	This study
30-8-1	1/2a	II	10	1030	17-Mar-05	15	Salmon roe "sujiko"	–	–	This study
30-11-1	1/2a	II	12	1039	17-Mar-05	2	Minced tuna	–	–	This study
30-29-1	1/2a	II	20	1035	17-Mar-05	11	Minced tuna	–	–	This study
32-27-1	1/2a	II	24	1045	14-Apr-05	10	Cod roe "mentaiko"	–	–	This study
36-6-1	1/2a	II	11	1039	2-Jun-05	2	Minced tuna	–	–	This study
36-17-1	1/2a	II	16	1039	2-Jun-05	24	Cod roe "tarako"	–	–	This study
36-25-1	1/2a	II	21	1039	2-Jun-05	22	Cod roe "tarako"	+	–	This study
36-25-2	1/2a	II	12	1053	2-Jun-05	22	Cod roe "tarako"	–	–	This study
37-1-1	1/2a	II	12	16619	9-Jun-05	25	Minced tuna	–	–	This study
37-3-1	1/2a	II	12	1053	9-Jun-05	25	Salmon roe "sujiko"	–	–	This study
38-16-1	1/2a	II	12	1039	16-Jun-05	19	Minced tuna	–	–	This study
38-16-3	1/2a	II	19	1030	16-Jun-05	19	Minced tuna	–	–	This study
39-2-1	1/2a	II	16	1039	21-Jul-05	24	Cod roe "tarako"	–	–	This study
40-4-1	1/2a	II	9	1030	26-Jul-05	25	Cod roe "tarako"	–	–	This study
40-6-1	1/2a	II	12	16619	26-Jul-05	2	Minced tuna	–	–	This study
22-19-2	3a	II	20	1035	16-Nov-04	11	Cod roe "mentaiko"	–	–	This study
22-28-5	3a	II	12	16619	16-Nov-04	12	Cod roe "mentaiko"	–	–	This study
26-2-3B	3a	II	12	1039	13-Jan-05	10	Cod roe "mentaiko"	–	–	This study
26-23-2	3a	II	20	1035	13-Jan-05	15	Cod roe "mentaiko"	–	–	This study
26-29-2	3a	II	12	1053	13-Jan-05	14	Cod roe "mentaiko"	–	–	This study
30-25-1	3a	II	23	1035	17-Mar-05	11	Cod roe "tarako"	–	–	This study
34-9-1	3a	II	17	1045	28-Apr-05	20	Cod roe "tarako"	–	–	This study
34-26-1	3a	II	20	1035	28-Apr-05	22	Cod roe "mentaiko"	–	–	This study
34-29-1	3a	II	9	1030	28-Apr-05	22	Cod roe "mentaiko"	–	–	This study
39-9-1	3a	II	12	16619	21-Jul-05	25	Cod roe "tarako"	–	–	This study
39-9-2	3a	II	9	1030	21-Jul-05	25	Cod roe "tarako"	–	–	This study
39-23-1	3a	II	20	1035	21-Jul-05	18	Cod roe "mentaiko"	–	–	This study
40-4-4	3a	II	12	1053	26-Jul-05	25	Cod roe "tarako"	–	–	This study
13-19	1/2b	I	2	1027	5-Nov-03	8	Cod roe "tarako"	–	–	Handa et al. (2005)
23-4-1	1/2b	I	1	1042	25-Nov-04	13	Salmon roe "sujiko"	–	–	This study
25-4-3	1/2b	I	1	1042	9-Dec-04	15	Salmon roe "sujiko"	–	–	This study
29-10-1	1/2b	I	4	1051	17-Feb-05	17	Minced tuna	–	–	This study
29-13-1	1/2b	I	5	1051	17-Feb-05	18	Cod roe "mentaiko"	–	–	This study
40-5-1	1/2b	I	2	1052	26-Jul-05	25	Salmon roe "sujiko"	–	–	This study
9-17	3b	I	3	1042	2-Feb-03	4	Salmon roe "sujiko"	–	–	Handa et al. (2005)
39-8-1	3b	I	2	1052	21-Jul-05	25	Salmon roe "sujiko"	–	–	This study
20-5-1	4b	I	6	1042	28-Oct-04	1	Cod roe "tarako"	–	–	This study
34-18-2	4b	I	7	1042	28-Apr-05	26	Cod roe "tarako"	–	–	This study

A total of 49 out of 59 *L. monocytogenes* isolates were collected from 531 ready-to-eat raw seafood retail products obtained at 61 different grocery stores in and around Tokyo between October 2004 and July 2005.

with an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using the same primers as were used for PCR amplification (Zhang et al., 2004). For each locus, alleles differentiated by at least one nucleotide were arbitrarily assigned different allele numbers (Maiden et al., 1998). Obtained sequences were deposited in the DNA Data Bank of Japan (DDBJ; National Institute of Genetics, Shizuoka, Japan) under accession numbers AB276438 through AB276791. Full sequences of *prfA* were deposited, but only positions 61–529 were used for MLST analysis.

2.5. Ribotyping

Automated ribotyping was carried out using a RiboPrinter microbial characterization system (DuPont Qualicon, Wilmington, DE, USA) according to manufacturer's instructions. Briefly, each isolate was streaked onto BHI agar plates, and following the overnight incubation at 30 °C, the appropriate amount of colonies were added to tubes containing sample buffer for cell lysis. Then the tubes were inserted into the RiboPrinter. This automated typing instrument employed *Eco*RI digestion of *L. monocytogenes* chromosomal DNA followed by Southern hybridization with an rRNA gene probe. Images were analyzed using RiboPrinter analysis software that normalized fragment pattern data for band intensity and band size relative to molecular weight markers, and were compared to database images for characterization. When an obtained ribotype pattern matched any one stored in the database with similarity of 0.85 or above, Dupont ID (e.g., DUP-1056) was automatically assigned.

2.6. Sequencing of complete inlA and prfA

The regions that contained complete sequences of *inlA* and *prfA*, respectively, and their flanking regions were amplified in all 59 *L. monocytogenes* seafood isolates so that direct sequence analysis could be performed. DNA sequencing was performed with an ABI Prism 3100 (Applied Biosystems) and the obtained sequences were aligned with GENETYX-WIN software (Genetyx, Tokyo, Japan). The amplification and sequence primers are shown in Table 2. The obtained sequences have been deposited in the DDBJ under accession numbers AB276379 to AB276437 for *inlA* and AB276438 to AB276496 for *prfA*.

Table 2
Amplification and sequencing primers used for *inlA* and *prfA* analysis

Gene	Primer name	Sequence (5'-3')	Product size (bp)
Amplification[a,b]			
inlA	inlA-F1	AATCCTATACAACGAAACCTGA	2490–2499
	inlA-R1	ATATAGTCCGAAAACCACATCT	
prfA	prfA-F1	TGTTGTTACTGCCTAATGTTTT	951
	prfA-R1	ACTCCATCGCTCTTCCAGAA	
Sequencing			
inlA	inlA-F2	TTTAAATCGGCTAGAACTATC	
	inlA-F3	AAGATATTAGCCCAATTTCT	
	inlA-F4	ATGCCTGCTAAAAACATCACC	
	inlA-R2	GGTGATGTTTTTAGCAGGCAT	
	inlA-R3	ATTTTTCACCGTGTTTGGA	
	inlA-R4	GATAGTTCTAGCCGATTTAAA	
prfA	prfA-F2	TTTAATGATTTTTCGATTA	
	prfA-R2	TAATCGAAAAATCATTAAA	

[a] For both *inlA* and *prfA* amplification, Mg^{2+} concentration was 1.5 mM, primer concentration was 1 mM, annealing temperature was 55 °C, and number of PCR cycles was 30.

[b] Amplification primers were also used as sequencing primers.

Table 3
Invasion efficiency of *L. monocytogenes* strains

Group	Isolates	Invasion %±SD[a]	Reference
1 Non-mutated *inlA*	EGDe	0.26±0.05	Nightingale et al. (2005a); Rousseaux et al. (2004)
	Scott A	0.64±0.17	Rousseaux et al. (2004)
	20-7-1	0.37±0.04	This study
	25-4-3	0.14±0.06	This study
	38-16-3	0.19±0.01	This study
	40-5-1	0.29±0.04	This study
2 Nonsense-mutated *inlA*	F2-563	0.05±0.004	Nightingale et al. (2005a)
	36-25-1	0.08±0.02	This study
3 *inlA* with 9 nucleotide deletions	20-5-1	0.18±0.04	This study
	34-18-2	0.30±0.07	This study

[a] The invasion rate was calculated as the number of bacteria recovered divided by the number of bacteria inoculated × 100.

2.7. Caco-2 cell invasion assay

Early confluent cell monolayers of Caco-2 cells (ECACC No. 86010202) were prepared using the Biocoat HTS Caco-2 assay system (Beckton Dickinson) following the manufacturer's instruction. The cells were seeded onto fibrillar collagen-coated wells at a density of 2×10^5 cells/well and incubated for 24 h in DMEM-based Basal Seeding Medium supplemented with MITO-Serum Extender (DMEM-MITO). After aspirating the medium, 500 µl of Entero-STIM Medium supplemented with MITO-Serum Extender was added to each well and incubated for 48 h. *L. monocytogenes* strains were selected for this invasion assay from 3 different groups based on the mutation type of *inlA* gene (Table 3). As control strains, the group of non-mutated *inlA* included EGDe and ScottA and the group of nonsense-mutated *inlA* included F2-563 since their high (EGDe and ScottA) or low (F2-563) invasion abilities were previously reported (Nightingale et al., 2005a; Rousseaux et al., 2004). Other strains included in the group of full length of InlA were selected randomly from strains listed in Table 1. After growing at 30 °C in brain heart infusion broth, *L. monocytogenes* cells resuspended in DMEM-MITO were added to infect Caco-2 cells. Following 2 h of incubation at 37 °C, bacterial cells that did not adhere to Caco-2 cells were washed away with PBS. The cells were incubated at 37 °C for 1 h in 500 µl of DMEM-MITO including gentamicin (50 µg/ml) to kill extracellular adherent bacteria. The cells were washed 3 times with PBS and lysed by maintaining them for 10 min in cold PBS containing 1% tritonX 100. The number of viable bacteria released from the cells was counted on TSAYE

plates by appropriate dilutions. Each bacterial strain was tested in triplicate.

3. Results and discussion

In a previous study (Handa et al., 2005), enrichment of this organism in food samples followed by isolation on selective agars and identification with a RiboPrinter microbial characterization system (Bruce, 1996) showed that the contamination rates among these samples were 10.0–17.1%, which are relatively high compared to those of most ready-to-eat foods, including cheese, meat products, and vegetables sampled in other countries (Farber, 2000; Gombas et al., 2003; Hitchins, 1996; Soriano et al., 2001). This survey indicates frequent exposure of Japanese people to this pathogen and shows that urgent risk assessment of ready-to-eat seafood products is necessary. Therefore, we specifically selected these types of foods for our study samples.

In our study, *inlA* and *prfA* were successfully amplified in all 59 of the *L. monocytogenes* seafood isolates, and the sequence analysis revealed that 58 out of 59 isolates and all 59 isolates lacked the nonsense codon producing truncated forms of InlA and PrfA, respectively. Our results are consistent with those of Jacquet et al. (2004) in that all strains of serotypes 1/2b and 4b did not have any nonsense codon in the sequence of *inlA*. On the other hand, only one out of 36 isolates of serotype 1/2a had a nonsense mutation in *inlA*, while 37% of food isolates of this serotype had truncated InlA according to Jacquet et al. (2004). Only strain 36-25-1 had a nonsense mutation at position 526, where adenine was converted into thiamine, resulting in a codon change from lysine into a nonsense codon TAA. This is the first report of a nonsense mutation at this position, while nine other different nonsense mutation positions have been previously reported (Jonquières et al., 1998; Nightingale et al., 2005a,b; Olier et al., 2002; Rousseaux et al., 2004). This suggests that there could be other positions with nonsense mutation resulting in the truncation of InlA, making it difficult to establish an easy method to detect strains with truncated forms of InlA, although this kind of technique would be a particularly useful tool for the risk assessment of foods. The invasion efficiency of this strain with the nonsense mutation was tested by performing a Caco-2 cell invasion assay. The greatly reduced invasion efficiency of this strain compared to those of strains with non-mutated *inlA* was confirmed as expected (Table 3).

In addition to the identification of a strain with a nonsense mutation in the *inlA* sequence, we identified two other isolates, 20-5-1 and 34-18-2, having 9-nucleotide deletions in the membrane anchor region (nucleotides 2212 to 2220 of *inlA*). This type of deletion has never been reported before for *L. monocytogenes* strains of any origin to the best of our knowledge. This type of deletion may have no effect on anchoring of the protein to the bacterial surface since the LPXTG motif, which allows a covalent linkage of the protein to the cell wall peptidoglycan, is retained (Navarre and Schneewind, 1994; Schubert et al., 2002). However, statistical analysis (*t* test) showed no significant difference between these two isolates and isolates with nonsense-mutated *inlA* in terms of invasion efficiency (P value = 0.104) (group 2 and 3 in Table 3). Furthermore, the invasion efficiencies of these two isolates were not significantly different from those of the isolates of non-mutated *inlA*, either (P value = 0.234) (group 1 and 3 in Table 3), whereas there was a significant difference between the invasion efficiencies of strains of groups 1 and 2 (P value = 0.0093). Even though these two isolates having 3 amino acid residue deletions seemed to be closer to the isolates with non-mutated *inlA* than to the isolates with nonsense-mutated *inlA* based on the statistics, this needs to be confirmed by testing larger numbers of strains.

No case of seafood-borne listeriosis has been detected in Japan until now (Makino et al., 2005). According to Jacquet et al. (2004), 35% of food isolates of no less than four different serotypes analyzed, including seafood isolates, had truncated InlA. Other reports have also shown that truncation of this protein is not a rare event among food isolates (Jonquières et al., 1998; Nightingale et al., 2005a). Therefore, it was unexpected that just one out of 59 isolates collected from 531 ready-to-eat raw seafood retail products distributed in and around Tokyo was mutated to have a truncated form of InlA in our study. The role of InlA was previously evaluated in virulence toward chick embryos by Olier et al. (2005). They compared the virulence between wild type strains and mutants constructed by allelic exchange of the *inlA* region, providing evidence of the necessary, but not sufficient, role of InlA in in vivo infection. In fact, strains having a full-length InlA have been isolated from a healthy child (Olier et al., 2002) and strains having a truncated form of InlA have been isolated from clinical patients (Jacquet et al., 2004), indicating that other factors contribute to virulence attenuation or induction. Therefore, even though almost all of the investigated seafood isolates had non-mutated *inlA*, this does not directly equate with full-virulence of these strains. We need to conduct further research to determine whether these isolates are fully virulent, and if not, which gene(s) contributes to virulence attenuation.

Nonsense-mutated *prfA* was not detected in all 59 isolates in this study (Table 1). To the best of our knowledge, there is only one report of PrfA truncation, and in it, all three isolates with truncated PrfA were found to have nonsense mutations at the same position (Roche et al., 2005). The truncated form of this protein may be more prevalent, and there may be other positions with nonsense mutation resulting in the truncation of PrfA, as in the case of the *inlA* gene. Since *prfA* is important in *L. monocytogenes* virulence because of its regulatory function over several virulence determinants, further investigation of nonsense-mutated *prfA* prevalence is needed.

Among the seafood isolates we analyzed, their distribution among the serotypes was as follows: serotype 1/2a, 61.0%; 3a, 22.0%; 1/2b, 10.2%; 3b, 3.4%; 4b, 3.4% (Table 1). Isolates of serotypes 1/2a and 3a comprised 83% of all isolates, and all of them belonged to lineage II, which is more associated with food isolates than human or animal isolates. Most of the ribotypes we obtained from these isolates were widely prevalent among many kinds of ready-to-eat foods (Gray et al., 2004) and no ribotypes from those common to outbreak isolates, such as 1038 and 1042 (Jeffers et al., 2001), were found. Moreover, no specific patterns

for these seafood isolates were detected in MLST when compared to other food isolates (data not shown). These subtypings reveal that the seafood isolates we analyzed were not especially virulent ones, making our results of *inlA* sequences more surprising.

Seafood isolates of serotype 1/2c were not isolated from 531 seafood samples investigated in this study, and thus, were not included in this analysis. This is consistent with previous reports showing that strains of this serotype were frequently isolated from meat products (Fantelli and Stephan, 2001; Farber and Peterkin, 1991; Johnson et al., 1990; Thévenot et al., 2005), and from seafood products with much less frequency (Dauphin et al., 2001; Handa et al., 2005; Johansson et al., 1999; Nakamura et al., 2004). However, the reason for these observations remains unknown since the primary source of food contamination is most likely the processing environment, rather than the raw material itself (Kathariou, 2002), and the difference in biofilm formation ability among different serotypes is still under discussion (Borucki et al., 2003; Djordjevic et al., 2002; Lunden et al., 2000; Norwood and Gilmour, 1999). In a survey of the prevalence of strains with nonsense-mutated *inlA*, we found that all 4 meat isolates of serotype 1/2c that we analyzed in parallel with the fish isolates had a nonsense-mutated *inlA*. This is consistent with results previously reported (Jacquet et al., 2004). As an epidemiological study of strains of serotype 1/2c, a larger group of isolates of this serotype may be needed to ascertain this finding. Also, elucidation of the low incidence of *L. monocytogenes* of serotype 1/2c in seafood isolates is needed to contribute to progress in food safety.

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