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教育講演 Ⅶ

遺伝子組換え乳酸菌を用いた経口粘膜ワクチン開発の試み

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要旨

これまでのワクチン研究から、病原体の感染経路からワクチンを投与することは非常に高いワクチン効果が期待されると考えられている。この考えからすると、腸管感染症のワクチンは、経口投与型粘膜のワクチンにより、最も有効な免疫効果が得られると考えられる。経口ワクチンでは、ワクチン投与により粘膜局所に誘導される分泌型IgAの働きによって、感染初期の病原体の体内への侵入阻止に機能する。経口ワクチンでは、全身性免疫の誘導も可能であり、これにより初期の粘膜からの侵入阻止を逃れて体内に侵入してしまった病原体の排除にも機能する。この論文では、これまで我々の行ってきた遺伝子組換え乳酸菌を抗原運搬体とする経口ワクチンの開発研究の結果を中心に紹介しながら、このワクチンの現状についてまとめてみる。

1．粘膜ワクチン研究

感染症の予防におけるワクチンの果たす役割はよく知られているが、理論的にその有用性が期待されながら実用化の遅れているワクチンが、経口粘膜ワクチンである。実験動物のレベルの研究では、以前から腸管粘膜局所における免疫の有用性が示されているが、その効果を十分に実現できる粘膜ワクチンの実用化は遅れている。

一般に、ある感染症に対するワクチンはその感染症の感染経路と同一な経路から投与するのが最も効果的であると考えられている。すなわち、感染経路が腸管粘膜である感染症を予防するワクチンは、経口投与による腸管粘膜からの粘膜免疫が最も高い効果が期待される。粘膜からのワクチン投与により、腸管粘膜局所における特異的な分泌型IgA抗体産生の増強による病原体の侵入ブロックが得られる。さらに粘膜ワクチンは全身性免疫の誘導も可能であり、粘膜局所の免疫に加え、二重の感染防御が期待できるワクチンである（図1)。

病原体そのものを用いた粘膜ワクチンの検討では、死菌体の経口投与では粘膜局所への免疫効果は一般的に充分ではないため、弱毒の生菌ワクチンが検討された。経口ワクチンは、消化管というワクチンにとっては最も過酷な環境で抗原性を保つ必要性から、これまで弱毒生菌ワクチン或いは人工的な抗原運搬体と感染防御抗原の組み合わせで検討されてきた。病原体を用いた弱毒生菌ワクチンの開発は、安全性の面からなかなか実用化に至らない場合が多い。

サルモネラなどの弱毒株を抗原運搬体とした遺伝子組換えワクチンは、マウスでのワクチン効果は非常に高かったが、安全性の面からヒトへの実用化はなかなか進まなかった。実用化を考えると、安全な抗原運搬体と感染防御抗原を組み合わせたコンポーネントワクチンにより粘膜ワクチンの作出が望まれている。腸内環境に適する運搬体と、遺伝子レベルで無毒化を行った感染防御抗原との

組み合わせで、ワクチンを構築する。人工粒子による抗原運搬体が検討されたが、こちらはワクチンの製造コストの高いことが指摘された。組換え植物や組換え酵母も抗原運搬体として検討されているが、免疫効果については充分とは言えない。

2. 乳酸菌を抗原運搬体とする組換えワクチンの開発

乳酸菌々体には、免疫賦活作用があることが知られており、その機能に関して、現在分子レベルの解析が始まっている1)。この機能は抗原運搬体としての利点となる。たとえば、サルモネラワクチンの投与に乳酸菌を加えて接種したところ、免疫効果が増強される場合があり、株によってその効果が異なっていたという報告2)がある。乳酸菌の中には、菌体自身に強いTh1型の免疫を誘導する作用を持つ株や、分泌型IgAの誘導作用の高い株がある。これらの機能は、まだそのメカニズムが解明されていないが、近年急速にその研究が進められている。

Lactobacillus casei では、Th1型の免疫反応によりインターフェロン(IFN)γを誘導する効果のある株が知られている。Th1型の免疫反応を誘導する乳酸菌は、IL-12を動かし、IFNγ産生を誘導することが知られている3)。このようなIFNγ誘導作用を持つ乳酸菌を抗原運搬体として用いれば、ウイルスや細胞内寄生菌など、細胞性免疫の誘導が感染防御に必要な微生物のワクチンとして有用である。一方、*Lactobacillus reuteri* では、株により、Th1型またはTh2型のいずれかの免疫反応に関係するサイトカインの誘導を促進する株がそれぞれ知られている。Th1型、Th2型の免疫誘導が菌株レベルで異なっていることは興味深い。Th2型の免疫反応を起こす乳酸菌では、IL-4やIL-10などのサイトカイン産生を高める。このような菌株を用いると、毒素の中和抗体や、病原体の進入を阻止する抗体の産生を誘導する目的に有用と思われる。このような乳酸菌々体の持つ免疫賦活効果と組み込む抗原の組み合わせを選ぶことにより、ワクチンを構築すると、様々な目的に適するワクチンが作出可能となる。

アジュバント効果以外についても、乳酸菌株の優位性は検討されており、*Lactobacillus plantarum* では、ヒトの胃における強い酸性に耐え腸管内での生残性が高い事が知られており、この特性は経口ワクチンの抗原運搬体として適当であると思われる。*Lactobacillus johnsonii* の抗原運搬体としての優位性を示す論文4)も報告されている。このような機能を持った乳酸菌株を、経口粘膜ワクチンの抗原運搬体として用いることは有望であると思われる。

3. 抗体産生を誘導する組換え乳酸菌ワクチン

感染に重要な働きをする抗原に対する特異的な抗体産生を誘導すると、感染防御が期待できる。例えば、毒素の細胞への結合に関わるエピトープに対する特異的抗体の誘導により、毒素活性を抑えることが可能である。また、微生物の細胞への接着侵入に関わるタンパクに特異的な分泌型IgA抗体を腸管粘膜局所に誘導すれば、微生物の細胞や体内への進入を阻止することが出来る。

我々は、腸管出血性大腸菌の腸管細胞への定着に関わるintiminのC末側をコードする遺伝子を組み込んだ組換え乳酸菌を作出し、マウスに投与し、この抗原に特異的な分泌型IgAを産生させることに成功した。この組換え体投与により、腸管出血性大腸菌の腸管細胞への定着を阻止することが期待される。

腸管出血性大腸菌の臨床症状の主要な原因となるのは、この菌の産生するShigatoxin（ベロトキシン）である。この毒素は、AとBの2つのサブユニットからなりAが毒素活性の本体、Bは毒素の細胞への結合に関わっていることが示されている。Bサブユニットは単独では毒素活性がなく、Bサブユニットに特異的な抗体は、その毒素活性を阻止することが報告されている。そこで、Bサブユニットをコードする遺伝子を乳酸菌に組み込みワクチンを作出した。この乳酸菌組換え体はB

サブユニットの結合に関する部分に特異的な抗体の産生を誘導した。Bサブユニットは遺伝子レベルで無毒化されており、組換え乳酸菌には、毒素活性は認められなかった。

これらの組換え乳酸菌を組み合わせて免疫を行うと、まずintimin特異的な分泌型IgA抗体により、腸管出血性大腸菌は、腸管内の定着が困難となり、感染のリスクは低下する。更に何らかの原因で体内に侵入してしまった腸管出血性大腸菌がShigatoxinを産生したとしても、毒素に特異的な血中IgGにより、毒素活性は抑えられる。このように腸管出血性大腸菌に対して２段階の感染防御が期待される。

腸管において粘膜上の抗体産生は細菌などの生きている細胞が有効であるという報告5)がある。その報告に従えば、乳酸菌を抗原運搬体とする組換えワクチンのような生きた微生物を利用したワクチンは効果が高いといえる。腸管の粘膜局所での抗体産生を期待する粘膜ワクチンでは、その抗原運搬体として主にTh2型のサイトカイン誘導能の高い乳酸菌を抗原運搬体として用いるとより強い効果が期待できる。*Lc. lactis* IL1403株を抗原運搬体とした豚丹毒菌のワクチンではマウスのレベルで有効な免疫効果が得られた6) (図2)。このワクチンでは、豚丹毒菌の感染防御抗原であるSpaAに対する特異的抗体が感染防御に関わっている。

4. 胞性免疫を誘導する組換え乳酸菌ワクチン

ウイルスや一部の細胞内寄生性の細菌では、抗体による感染阻止は困難であり、その感染防御に細胞性免疫を必要とする。このような微生物に対しては、Th1型の免疫を誘導する乳酸菌に、感染防御抗原をコードする遺伝子を組み込み発現する必要がある。HIVのワクチンとして、*Lc. lactis*を抗原運搬体とし、コレラトキシンをアジュバントとして用い、マウスで十分な免疫効果が得られたと報告されている7)。この研究では、乳酸菌として*Lc. lactis* IL1403株を用いている。この株は、菌体にTh1やTh2といったはっきりとしたサイトカイン誘導効果はなく、いわゆる中性的な抗原運搬体である。この抗原運搬体をTh1型のCTL誘導型の乳酸菌とすることによりさらに強い免疫効果が期待できるのではないかと思われる。この場合、実験に用いていたコレラトキシンのアジュバント効果を必要としない程度に免疫増強が起これば、実用的なワクチンとして期待できる。Th1型の乳酸菌を用いたSARSのワクチンが報告されている8)。

我々は、細胞内寄生性でその感染防御に細胞性免疫を必要とするリステリアに対するワクチンを作出した。*Lactobacillus casei* ATCC393株は、Th1誘導作用が認められる。そこでこの株に、リステリアの最も主要な病原因子であるlisteriolysin O (LLO)をコードする遺伝子を組み込み、組換え乳酸菌を作出した。この遺伝子は、乳酸菌との相性が良く、乳酸菌々体表層への発現は良好であった。この組換え乳酸菌をマウスに経口投与し、致死量のリステリア強毒株を腹腔内投与しても、マウスは生存する。経口投与によりマウスの血液中のIFNγ産生を誘導し、感染防御に機能する細胞性免疫が誘導されたことを実証したことは画期的であり、現在さらに効果の高いワクチンとなるよう研究中である。このように乳酸菌組換えワクチンは、既に医薬品に相当する機能を持ちつつある。

5. 生産動物用のワクチン開発

Salmonella Enteritidis食中毒の原因食品は、そのほとんどが卵およびその加工品である。本菌が卵の内部を汚染(in egg汚染)することにより、食中毒の原因となる。本菌の食中毒は、不顕性感染を起こした親鳥が、卵の内部に本菌の汚染を受けた卵を産卵することにより発生する。従って本菌による卵の汚染を排除するには、産卵後は困難で、産卵鶏がサルモネラに感染しない状況を作ることが最も重要である。現在は、飼育環境をクリーンにする、飼料中に抗菌物質を加える、プロバイオティクスを投与する、産卵鶏にサルモネラのワクチンを投与する等の対策が取られている。

特に、プロバイオティクスの継続的な投与と、サルモネラのワクチン投与が注目されている。現在用いられているサルモネラワクチンは、サルモネラ死菌体のワクチンであるが、将来的には、乳酸菌を抗原運搬体とした経口ワクチンへの切り換えが考えられる。我々は、サルモネラの主要な感染防御抗原であるFli C（鞭毛抗原）を菌体表層に固定化した組換え乳酸菌ワクチンを作出し、マウスに於いて、感染防御効果が得られることを示した9)（図3）。現在産卵鶏を用いてその効果を調べている。このワクチンの投与により産卵鶏は、サルモネラ感染を起こさなくなり、本菌の卵の中への汚染が阻止されることが期待される（図4）。

6．今後の研究

これまで述べてきたように、遺伝子組換え技術により、乳酸菌は経口ワクチンの抗原運搬体として新しい機能を獲得する事ができる。乳酸菌はプロバイオティクスとしての機能を期待され、遺伝子組換えによりさらに高い効果を期待される。乳酸菌組換えワクチンは、既に医薬品に相当する機能を持ちつつあり、その高い機能ゆえ、一部の組換え体では医薬品と同等の管理を必要とする可能性がある。また、機能を高めたプロバイオティクスの実用化には誰もが納得できるような遺伝子組換え体の安全性の議論も必須である。乳酸菌の育種の方法としての遺伝子組換えは、やっと本格的に始まったところである。これからも新しい機能や利用方法が出てくると思われる。たとえば、嫌気性組換え乳酸菌を用いたガン治療が、臨床実験を計画している。組換え乳酸菌を利用したアレルギー治療剤の開発研究が開始されている。乳酸菌に機能を持った物質をコードする遺伝子を組み込み、生体内での生産工場として利用するといった試みもされている。たとえば、サイトカインをコードする遺伝子を乳酸菌に組み込み生体内で発現させるといった研究 10)である。これらもよく考えてみると、今回説明してきた経口ワクチン開発と同様、乳酸菌の基本的な遺伝子組換え技術の利用により、プロバイオティクス機能の強化や新しい機能の獲得の延長線上にあるものである。

これまでは、乳酸菌への組換えの応用は商業ベースではどちらかというとタブーであるかのごとく扱われてきた。今後は、組換えにより得られる乳酸菌の機能によるメリットと安全性を秤にかけて、乳酸菌における組換え技術の有用性を正当に評価し活用してゆくことになると思う。

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英文抄録

Development of oral vaccines based on lactic acid bacteria

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Based on previous studies of vaccination, it is recognized that the administration of a vaccine through the infection route of a pathogen is highly effective for vaccination. According to the concept, oral vaccination is the best strategy for inducing immune responses against enteric infections. Oral vaccines induce mucosal immune responses mediated predominantly by secretory immunoglobulins A (sIgA), which serve as the first line of defense against the pathogens. Oral vaccines also elicit systemic immunity, which ensures protection against the pathogens in case they have escaped the first line of defense on the mucosal surfaces. In this paper, I introduce our challenge to develop effective oral vaccines based on recombinant lactic acid bacteria as antigen delivery vehicles

図1

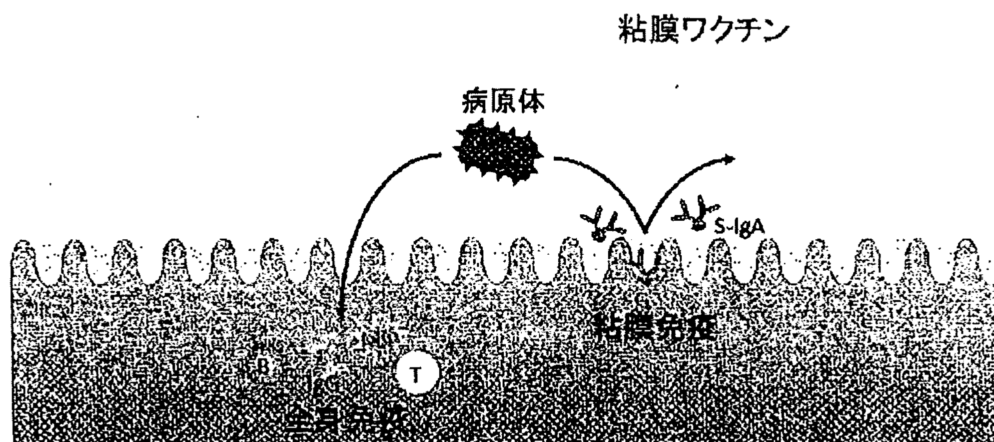
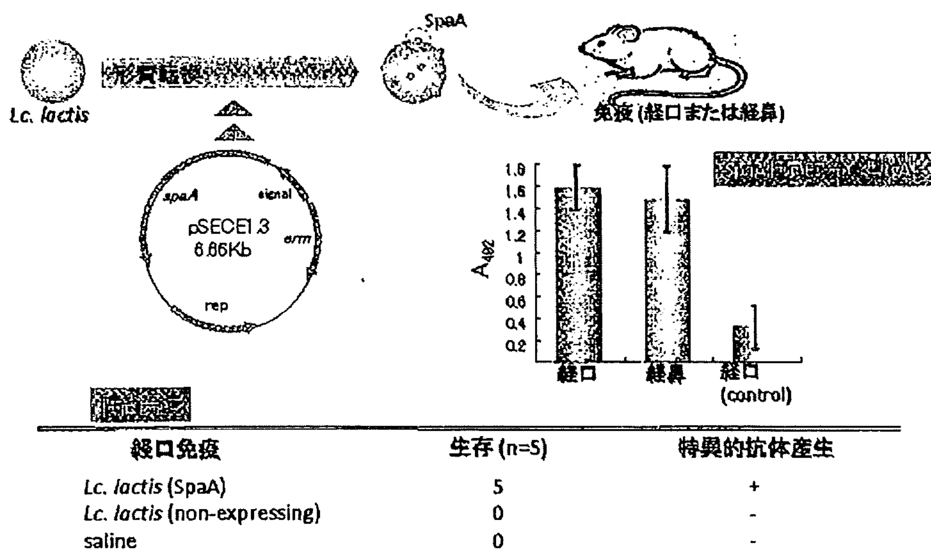


図1. 経口粘膜ワクチン
経口粘膜ワクチンは経口的に投与し、粘膜局所免疫と全身性免疫の両方の免疫を誘導し感染を防ぐワクチン

図2



経口免疫	生存(n=5)	特異的抗体産生
Lc. lactis (SpaA)	5	+
Lc. lactis (non-expressing)	0	-
saline	0	-

図2. 豚丹毒ワクチンの感染防御効果
豚丹毒菌の表層タンパク質SpaAを組み込んだ乳酸菌組換えワクチンの免疫効果を示す。特異的抗体産生により、感染防御効果が認められる。
(Cheun HI, et al. 2004. (文献6)より改変)

図3

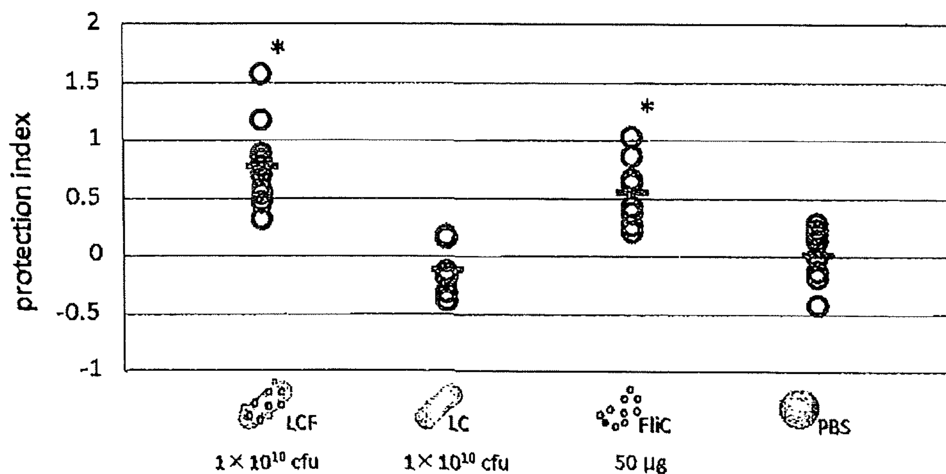


図3. FliCを組み込んだ組換え乳酸菌ワクチンのマウスにおける感染防御効果

protection index = $\log_{10}$ CFU $_{\text{PBS-mean}}$ − $\log_{10}$ CFU $_{\text{individual}}$

— mean value　　＊ $P<0.01$　　(Kajikawa et al. 2007. (文献9)より改変)

図4

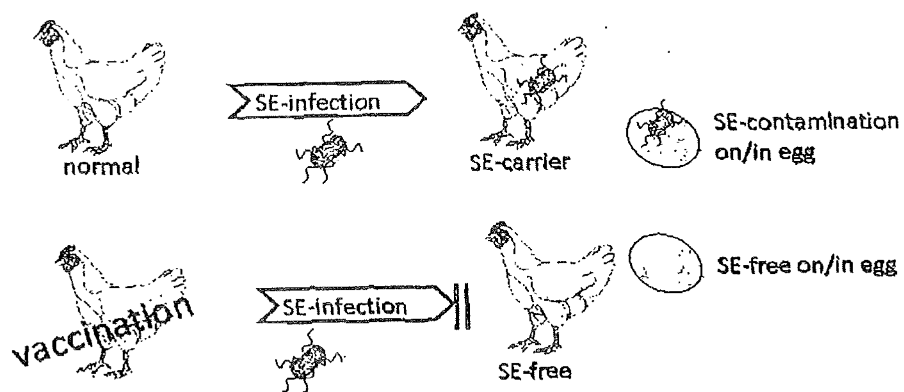


図4. サルモネラワクチンによる生産動物の微生物コントロール

Real-Time Polymerase Chain Reaction Method for Detecting Contamination of Beef by Material from Genetically Engineered Cattle

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Prion protein knockout (PRNP$^{-/-}$) cattle have been developed and may be used to produce bovine material such as serum, collagen, and gelatin. However, genetically engineered animals (GE animals) must not be imported or made commercially available in Japan, because they are not authorized for food use in Japan. We used real-time polymerase chain reaction (real-time PCR) to develop method of detection for neomycin- and the puromycin-resistance genes in beef samples. Plasmids containing the neomycin-resistance gene and the puromycin-resistance gene were used as standard reference molecules. The results clearly showed that the method we developed is capable of quantitatively detecting the neomycin- and the puromycin-resistance genes in the plasmids in the presence of genomic DNA extracted from a beef sample. We also applied the method to testing of beef samples imported from the United States (U.S.). This method will make it possible to monitor beef for contamination by material from GE cattle to assure food safety.

Key words prion protein knockout cattle; real-time polymerase chain reaction; beef

Many types of genetically engineered animals (GE animals) have been developed. In 2007, Richt *et al.* generated GE cattle in which two prion protein alleles had been disrupted (PRNP$^{-/-}$cattle) by inserting neomycin- and puromycin-resistance genes as selection markers and they discussed the possibility of using PRNP$^{-/-}$ cattle as a source of milk, gelatin, collagen, serum, and plasma.[1] Such products would be prion-free. Additional genetic modification against a PRNP$^{-/-}$ background could be useful for producing prion-free therapeutic recombinant human proteins, tissue, and reagents in transgenic livestock for biomedical application.[1] The use of PRNP$^{-/-}$ cattle appears very attractive if the animals are kept under appropriate management.

However, GE animals, including PRNP$^{-/-}$ cattle, have not been authorized as a source of food in many countries, and Japan has made safety assessment of genetically modified (GM) foods and processed foods containing GM ingredients mandatory. Since April 1, 2001, any GM food that has not been authorized is prohibited from importation or sale in Japan. If contamination of the food by material from unauthorized GE animals is suspected, the food must be recalled, thereby making it necessary to rapidly and accurately detect contaminating unauthorized GE material.

In the present study we used real-time polymerase chain reaction (real-time PCR) to develop a method of detecting materials produced from GE cattle. The method is based on the detection of neomycin- and puromycin-resistance genes inserted in the cattle genome as selection markers. Five samples of beef imported from the U.S. were then monitored using the present detection method.

MATERIALS AND METHODS

Materials Five samples of beef imported from the U.S.A. (four of non-processed beef, one of grilled beef), and a sample of beef produced in Japan (non-processed beef) as a negative control, were purchased at a market in Tokyo. As standard material for the calibration curves of real-time PCR, plasmids pcDNA 3.1(−) and pIRESpuro3 Vector were purchased from Invitrogen (Carlsbad, CA, U.S.A.) and Clontech (Mountain View, CA, U.S.A.), respectively.

Extraction of Beef DNA Genomic DNA was extracted from 2 g of each beef sample by using two columns of Genomic-tip 20/G (Qiagen, Hilden, Germany) according to the manufacturer's manual but with the modification described previously.[2] The DNA concentration in the prepared DNA solutions was determined by measuring UV absorption at 260 nm with a GeneQuant *pro* spectrophotometer (Amersham Biosciences, Piscataway, NJ, U.S.A.).

Real-Time PCR Detection of Neomycin- and Puromycin-Resistance Genes Real-time PCR was performed by using the ABI PRISM® 7900HT sequence detection system (Applied Biosystems, Foster City, CA, U.S.A.). All reactions were run in triplicate in 96-well plates. A no-template control (NTC) was also prepared as a negative control for the analyses. The data were analyzed by using the data analysis software version 2.1 included in the real-time PCR system. When the cycle threshold value was more than 38, we considered the amplification to be negative because of the difficulty of confirming the exponential amplification.

Calibration of Standard Curves for the Neomycin- and Puromycin-Resistance Genes in Real-Time PCR The PCR reaction mixtures were placed in a final volume of 25 μl consisting of 12.5 μl of the universal PCR master mix (Applied Biosystems), 0.75 μl of the primer pair (10 μM each), 0.5 μl of the *Taq*Man MGB probe (10 μM), and 2.5 μl of control plasmid template DNA. Standard curves were plotted by using seven concentrations of the control plasmid template DNA, *i.e.*, containing 20, 200, 2.0 k, 20 k, 200 k, 2.0 M, and 20 M copies, respectively.

For detection of the neomycin-resistance gene, pcDNA 3.1(−) digested with *Eco*RI was used as the template DNA, and the nucleotide sequences of the sense and anti-sense primers used were NeoF (5′-CGACCACCAAGCGAAACAT-3′) and NeoR (5′-CTCTTCGTCCAGATCATCCTGAT-3′), respectively. The probe structure was NeoPro, 6-carboxy-fluorescein (FAM)-CATCGAGCGAGCACGTA- minor groove binder (MGB).

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For detection of the puromycin-resistance gene, pIRESpuro3 Vector digested with *Eco*RI was used as the template DNA, and the nucleotide sequences of the sense and antisense primers used were PuroF (5′-TCACCGAGCTGCAAGAACTCT-3′) and PuroR (5′-CCCACACCTTGCCGATGT-3′), respectively. The probe structure was PuroPro, FAM-CCTCACGCGCGTCG-MGB. The reaction conditions included an initiation step for 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 10 s at 95 °C and 1 min at 60 °C.

Calibration of the Standard Curves in the Presence of DNA Extracted from a Beef Sample The genomic DNA extracted from the Japanese beef sample was added to the PCR reaction mixture to a final concentration of 10 ng/μl. A 1.25 μl volume of endogenous 18S ribosomal RNA (rRNA) primers and probe (No. 4319413E, Applied Biosystems) was added to the PCR reaction mixture as the internal control. The endogenous 18S rRNA probe was labeled with fluorescent reporter dye VIC, and duplex PCR was adopted. The control plasmids for the standard curves and the PCR reaction conditions were the same as described in the section above.

Survey of Samples of Beef Imported from the U.S.A. and Purchased in a Japanese Market Genomic DNAs extracted from samples of beef imported from the U.S.A., instead of the genomic DNA extracted from the Japanese beef sample were added to a final concentration of 10 ng/μl in the PCR reaction mixture described in the calibration of standard curves in the presence of DNAs extracted from a beef sample section. No control plasmids were added.

The experiments described in the calibration of standard curves in the presence of DNA extracted from a beef sample section were conducted in parallel to construct standard curves. The presence of the neomycin- and puromycin-resistance genes in samples of beef imported from the U.S.A. was examined with the same PCR reaction conditions described in the section above.

RESULTS

Extraction of DNA from Beef Samples The amounts of genomic DNA in the DNA sample solutions prepared from the non-processed beef samples were in the 63—117 μg range. The size of the extracted DNAs appeared to be mostly greater than 6.6 kb according to agarose gel electrophoresis analysis as shown in Fig. 1. The amount of genomic DNA in the DNA sample solution prepared from the grilled beef sample was 16 μg. The length of the DNA prepared from the grilled beef sample was in the 0.2—1.4 kb range. This result indicates that the genomic DNAs extracted from the grilled beef sample had been degraded by the processing.

Establishment of Calibration Curves We designed specific primers and probes for the neomycin-resistance gene and the puromycin-resistance gene based on their sequences. The primers and probes were then used to confirm that the amplification curves could be obtained with the pcDNA3.1(−) and pIRESpuro3 Vector series digested with *Eco*RI as standard molecules, and that neomycin- and puromycin-resistance genes could be detected quantitatively by using real-time PCR.

Next, the plasmids were spiked into genomic DNA extracted from the Japanese beef sample. We then confirmed that the amplification curves could be obtained with the plasmids and that the two resistance genes could be detected in the presence of genomic DNA extracted from a beef sample. In the tests for both resistance genes we also confirmed that the amplification curves of the probe labeled with the VIC dye for the endogenous 18S rRNA gene were obtained for all sample solutions to evaluate the quality of the extracted genomic DNAs. As shown in Fig. 2, the standard curve for the

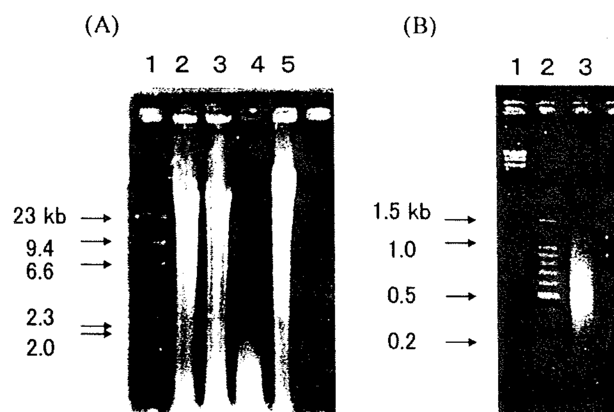

Fig. 1. (A) 0.8% Agarose Gel Electrophoresis of DNA Extracted from Beef Samples

Lane 1: DNA size marker, lanes 2, 3, 5: DNA extracted from samples of non-processed beef, lane 4: DNA extracted from a sample of grilled beef.

(B) 2% Agarose Gel Electrophoresis of DNA Extracted from a Beef Sample

Lane 1, 2: DNA size markers, lane 3: DNA extracted from a sample of grilled beef.

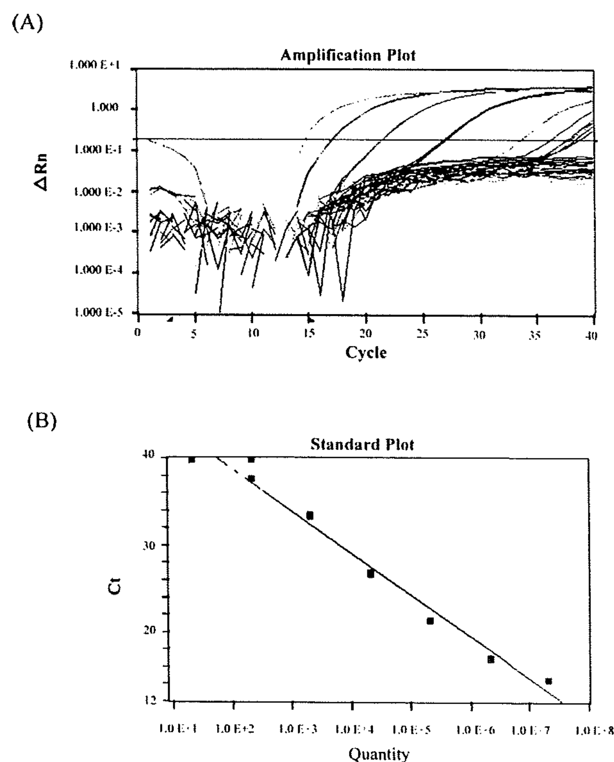

Fig. 2. Real-Time PCR Analysis of a Neomycin-Resistance Gene in the Presence of DNA Extracted from a Sample of Japanese Beef at a Concentration of 10 ng/μl

(A) The amplification plots. (B) The standard curve.

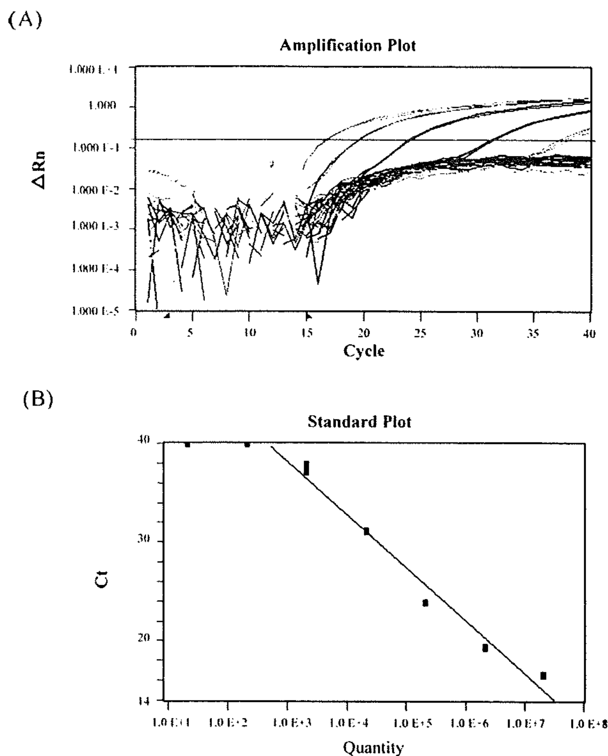

Fig. 3. Real-Time PCR Analysis of a Puromycin-Resistance Gene in the Presence of DNA Extracted from a Sample of Japanese Beef at a Concentration of 10 ng/μl

(A) The amplification plots. (B) The standard curve.

amount of the neomycin-resistance gene copy number was calibrated by using the pcDNA3.1(−) series digested with *Eco*RI: $y = -4.830x + 48.42$ (threshold 0.20, $R^2 = 0.979$). In addition, as shown in Fig. 3, the standard curve for the amount of the puromycin-resistance gene copy number was calibrated by using the pIRESpuro3 Vector series digested with *Eco*RI: $y = -5.366x + 54.25$ (threshold 0.16, $R^2 = 0.973$). We estimated that the detection limit was 144 copies for the neomycin-resistance gene and 1070 copies for the puromycin-resistance gene, based on the formulas and the levels at 38 as Ct values.

Survey of the Five Samples of Beef Imported from the U.S.A. To assess the validity of our method we used it to test five samples of beef imported from the U.S.A. The standard curves were used to determine whether the neomycin- and puromycin-resistance genes were present in the samples of beef imported from the U.S.A. Trace amounts of the neomycin-resistance gene were detected in one of the five beef samples. However, the results were not reproducible because the number of copies of the gene appeared to be close to the detection limit. The puromycin resistance-gene, on the other hand, was not detected in any samples. Analysis of the endogenous 18S rRNA gene yielded amplification plots similar from all five samples. In all of the tests for the endogenous 18S rRNA, the threshold lines were set at 0.20, and the Ct values were constant (between 16.8—18.9).

DISCUSSION

In this study we developed real-time PCR method for detecting the neomycin-resistance gene and puromycin-resistance gene inserted into the genome of GE cattle as a means of testing beef for the contamination by material from GE cattle.

Because we considered it difficult to obtain material that originated in $PRNP^{-/-}$ cattle as a positive control and to obtain a sequence that would be specific for $PRNP^{-/-}$ cattle, we used plasmids containing the neomycin-resistance gene and puromycin-resistance gene as alternative standard reference molecules to develop the detection method. The specific primer pairs and probes for the both resistance genes were designed within the sequences of each resistance gene for the real-time PCR experiments. The results clearly showed that the neomycin- and the puromycin-resistance genes could be quantitatively detected in the presence of genomic DNA extracted from beef samples, suggesting that the detection method is capable of being used to detect genomic DNAs from $PRNP^{-/-}$ cattle.

Many real-time PCR detection methods based on fluorescence detection, such as *Taq*Man® chemistry, have been developed to identify and quantify GM maize,[2—7] GM soybeans,[2—5,8,9] and GM varieties of other agricultural commodities[7] and to detect pork, beef, chicken, mutton, and horse in foods.[10] This model experiment in which standard plasmids were spiked into genomic DNA from a beef sample and were detected is thought to be reasonable and acceptable, because plasmid DNA markers containing cloned transgenic sequences have been used for genetically modified organism analysis.[3]

We applied this detection method to testing five samples of beef imported from the U.S.A., and trace amounts of the neomycin-resistance gene were detected in one of the five samples in our survey. Sequences homologous to the 3′-aminoglycoside phosphotransferase gene, which is responsible for neomycin resistance, have been detected in raw ground beef,[11] consistent with our own results. Since it is almost impossible to completely remove contaminating bacteria containing DNA sequences homologous to the 3′-aminoglycoside phosphotransferase gene from beef samples, we considered it important to simultaneously detect both the neomycin-resistance gene and puromycin-resistance gene as an appropriate criterion for a positive result. Based on this criterion all five samples of beef imported from the U.S.A. that we tested were concluded to be negative for contamination by material from $PRNP^{-/-}$ cattle.

The results of our tests confirmed that the genomic DNA extracted from the grilled beef sample was degraded, as expected. However, we concluded that the genomic DNA from the grilled beef was measurable in our real-time PCR analysis, because the amplification curves for the endogenous 18S rRNA reactions were clearly detected by testing of the genomic DNA from the grilled beef sample, the same as the genomic DNA from the non-processed beef samples, although the sensitivity of the test for the grilled beef sample would be lower.

The C-value for cattle is 3.15—3.93 according to Animal Genome Size Database.[12] In our study we used 250 ng of DNA extracted from each sample of beef imported from the U.S.A., and the cattle genome copy number would be approximately $6.36—7.94 \times 10^4$ copies per sample. Based on this number of copies and the detection limit of our method,

the target genes would be detected if the contamination ratio of the materials from GE cattle were 100%. In terms of the commercial process, beef material from GE cattle and from non-GE cattle might be mixed together in the future. Further study will be necessary to assess the feasibility of detecting lower levels of GE cattle material by using reference standards and extend the applications of the method we developed to more complex processed food products. It will be desirable to examine the appropriate way for the preparation of genomic DNAs from hamburger, canned beef, beef jerky and to apply the method to testing the genomic DNAs.

Furthermore, since both the neomycin- and puromycin-resistance genes are widely used as selection markers when mammalian cells are genetically modified, detection using the present method does not specifically mean that the material prepared from $PRNP^{-/-}$ cattle is contaminated. However, it would be suspected that the material derived from kinds of GE cattle could be contaminated. This detection would suggest that more detailed analyses would be necessary.

In conclusion, rapid detection method for neomycin-resistant genes and puromycin-resistant genes inserted in the GE cattle genome has been established. This method would monitor beef for contamination by material from GE cattle to assure food safety.

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