明と診断・治療精度を向上させるための研究，慢性の痛み診療の基盤となる情報の集約と，より高度な診療のための医療システム構築に関する研究を開始している[12]．

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▷第60回学術集会
シンポジウム4：国際標準からみた日本の臨床微生物検査における課題(5)◁

感染症診断および感染制御における新世代の遺伝子検査システムの臨床的意義

山岸由佳*1　三鴨廣繁*2

Clinical Significance of Next-Generation Molecular Testing Systems for the Diagnosis of Infectious Diseases and Infection Control

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The clinical significance of molecular testing for the diagnosis of infectious diseases and infection control has been increasing. Traditional molecular tests which required special pretreatment, such as nucleic acid extraction and pretreatment processes, have depended on manual methods. Therefore, different results were frequently observed between various methods and/or among facilities. In order to solve these problems, automated machines for nucleic acid extraction have been developed. Automated nucleic acid extraction systems, which automatize the nucleic acid separation and refinement processes, prevent contamination, reduce human errors, allow for stable processing, minimize differences among facilities, and promote the standardization of molecular tests. Rapid diagnosis of infectious diseases using various simplified molecular testing methods has led to increased success rates of infectious disease treatment and infection control measures by utilizing rapidity and accuracy. Herein, we introduce four automated molecular testing systems: Cepheid Xpert, BD MAX, Seegene kit, and Hologic Gen-Probe PANTHER system.【Review】

[Rinsho Byori **62**：000〜000, 2014]

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【Key Words】 molecular test(遺伝子検査), rapid test(迅速検査), automated machine(自動機器), polymerase chain reaction: PCR(ポリメラーゼ連鎖反応)

感染症領域における検査の歴史をひもとくと，19世紀に培養，グラム染色が普及し，20世紀に薬剤感受性試験や抗原検査が，そして2010年前後に分子生物学的検査として核酸増幅法やハイブリダイゼーション，シークエンス解析に基づく同定が目覚ましい進化を遂げている。また，同時にMatrix Assisted Laser Desorption/Ionization(マトリックス支援レーザー脱離イオン化法)(MALDI-TOF Mass Spectroscopyなど)を用いたプロテオミクス解析の臨床応用が進んでいる。このように昨今では種々の検査が実施可能となっているが，感染症治療における抗菌薬治療が遅れると時間依存的に生存率が低下する[1]と

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いう報告や，不適切治療では予後が悪いという報告[2)3)]がある。特に感染症診療においては，初期の経験的治療から培養結果報告，薬剤感受性結果報告のステップのうちどのタイミングが不適切な抗菌薬選択であっても患者予後に影響を与える[4)]。これらのことから，いかに早期に迅速に診断し，適切な抗菌薬投与を開始するかどうかが患者の生命予後に影響するかという事がわかっている。

近年，様々な遺伝子検査が登場し，冷蔵不要，ダイレクトサンプリング，オートメーション，可能な限り小さな機器，を可能としている。本稿では，近未来に日本でも実用化が期待されている自動化された代表的な遺伝子検査機器であるXpert®シリーズ(セフィエド社)，BD Max®(日本ベクトン・ディッキンソン社)，Anyplex™ IIシリーズ(Seegene社)，パンサー™システム(ホロジック社)の4つを紹介する。

I. GeneXpert®/Xpert®システム(セフィエド社)

A. 概　　要

セフィエド社のGeneXpert® System(Cepheid, USA)は，次世代の遺伝子診断法・機器として既に欧米や途上国に普及し，感染症分野において注目されているシステムである。項目毎に設計された専用試薬のXpert®カートリッジと共に使用され，核酸抽出，PCR増幅，検出を統合した自動遺伝子解析システムで，技術者に特別な訓練は不要で，数分の簡便な用手操作後，全自動で正確な結果を迅速に供することが可能である。Xpert®カートリッジを変更すれば，同一機器で様々な病原体に対応し，項目にもよるが32～120分で迅速に結果を得ることができる。日本国内ではGeneXpert®システムとして医療機器の届出がなされ，試薬は臨床性能試験が進行中で，近い将来日本市場でも利用可能となる見込みである。

B. GeneXpert® Systemとは

GeneXpert® Systemは自動遺伝子解析装置で附属のコンピュータに内蔵されている専用ソフトウエアで装置を制御している。独立稼働のGeneXpert®モジュールが1，2，4および16の4種類の装置(**Fig. 1**; GeneXpert® System VI)があり，専用のXpert®カートリッジ試薬内部で核酸抽出からリアルタイムPCR反応，検出および結果解釈までの全行程が自動的に行われる。Xpert®カートリッジは内部が11のスペースに分かれており，それ自体が閉鎖系の遺伝子検査室として機能している。**Fig. 2**に反応ステップを示すように，PCRに必要な凍結乾燥および液状試薬が充填され，高度なマイクロ流体工学技術によって試薬と検体の混和や液体の移送や核酸抽出とフィルター吸着を実行し，横断面状の反応チューブ内でPCR増幅反応後，最大6波長によるマルチプレックス解析を行う。用手操作は非常に簡単で，検体を直接添加する項目もあるが，キット添付の検体処理液で細胞溶解や不活化を数分ほど実施後，カートリッジの検体注入口に添加し，GeneXpert®モジュールにセットするのみである。各GeneXpert®モジュールは独立起動のため1テストずつ測定が可能で，オンデマンド遺伝子検査としての運用を実現する。また，GeneXpert®システムは，内部コントロールとしてB. globigiを採用したSPC(Sample processing control)を含み，カートリッジ毎に核酸抽出やPCR反応が適切であるかどうかを管理する機能や，確実な試薬溶解と適正な蛍光度を評価するProbe check controlも内蔵しており，高い精度を担保している。

Figure 1　GeneXpert system IV.

C. 感染症診療におけるGeneXpert® Systemの貢献

結核菌とリファンピシン耐性の有無を同時に検出できるXpert® MTB/RIFは，簡便，迅速および正確を評価し途上国向け優良システムとして世界保健機構(World Health Organization: WHO)の推奨[5)]を受け，全世界で広く普及している。喀痰から直接アッセイを行うことが可能で，約2時間で結果を供することができる。塗抹陽性・培養陽性となる検体は98%，塗抹陰性・培養陽性となる検体は68%の検出感度が報告[6)]されている。リファンピシン耐性の診断精度

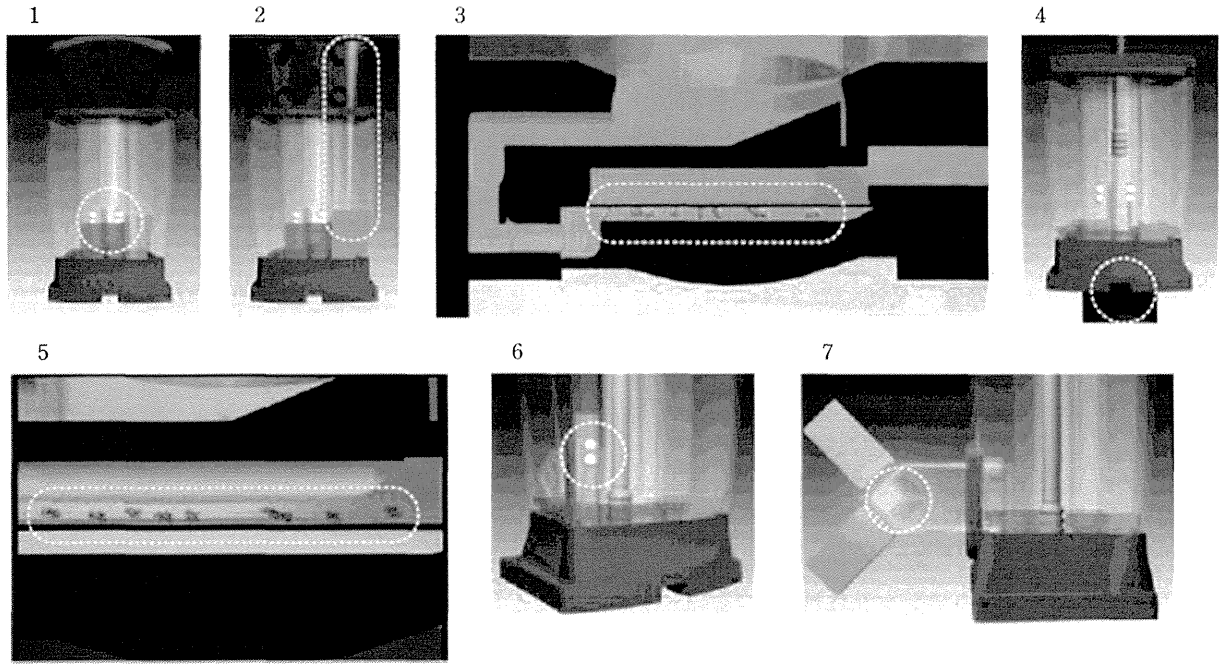

Figure 2　Xpert®カートリッジ試薬内の反応工程.
1. 充填済の試薬
2. 検体懸濁液を添加
3. フィルター上に捕獲された微生物
4. 超音波ホーンによる微生物粉砕と DNA 遊離
5. DNA がフィルターを通過し最初のチャンバーに移送
6. DNA は溶解された試薬と混和され反応チューブに注入
7. PCR 反応および検出

は感度 94%，特異度 98%とされ[6]，*rpoB* 遺伝子の RRDR 領域における変異を 5 種類のモレキュラービーコンプローブで検出する方法で，遺伝子変異がある場合はシグナル未検出によって変異があることで検出される。

医療関連感染で重要とされている *Staphylococcus aureus*，*Clostridium difficile* の遺伝子検査も，昨今注目されているが，鼻腔スワブ検体から *S. aureus*（SA）あるいは methicillin-resistant *S. aureus*（MRSA）を同定することが可能な Xpert SA Nasal Complete Kit は，ターゲットとして *spa* 遺伝子（*S. aureus* ProtainA），*mecA* および *SCC-orfX* junction 領域を採用している。外科手術後の感染症が増加している中で，特に *S. aureus* の感染は劇症になることがあるため，感染のリスクとなる保菌状態を予め確認し，除菌を行うことによって手術後感染症が減少したという報告[7]がある。また，術前スクリーニング後にムピロシン軟膏とクロロヘキシジンによる除菌を行うことによって，*S. aureus* 感染が約 60%減少し，入院日数が 2 日間短縮したという報告[8]では，予防対策費用がスクリーニング費用と軟膏の費用のみとなりコスト削減に貢献し，これらの予防対策費用に比べて治療費の方がはるかに高価であったと考察されている。

C. difficile の検出については，米国微生物学会（American Society for Microbiology）のガイドライン[9]において，酵素免疫法単独で *C. difficile* トキシン陽性を報告することは勧められておらず，GDH 陽性かつ EIA 法でのトキシン陽性，もしくは培養細胞毒素中和法での陽性または PCR 法での陽性確認後に陽性の報告をすることを義務づけている。一方，PCR 法は単一の検査法として *C. difficile* 検査に使用することが可能で，欧米では Xpert *C. difficile* が広く普及している。Xpert *C. difficile* は，トキシン B，バイナリートキシンおよび流行株 027 を検出することができ，*C. difficile* の検出には *tcdB*（Toxin B）のみ，バイナリートキシンは高度に保存された領域である

BDマックス™の特長

◆検体から直接，遺伝子検査を実施
→ 迅速・高感度に結果を得られることで，早期対策に有効

◆BDマックス™により核酸抽出・増幅・検出工程を全て自動化
→ 作業効率の向上，人為的ミスの削減が可能

◆MRSAや他の耐性菌，HAI原因微生物等の迅速検出
→ これからの更なる院内感染対策に有用

◆オープン試薬を用いて独自の反応系の構築が可能
→ 培養困難な微生物や毒素等の検出にも有用

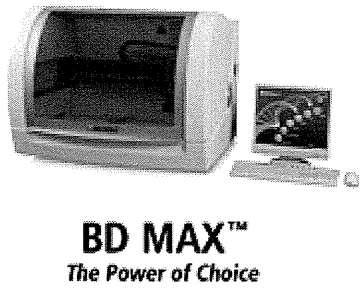

Figure 3

cdtA，027株は毒素産生を抑制する遺伝子*tcdC nt* 117の欠損部を，それぞれターゲット検出部位としている。水様あるいは泥状便をキット付属の検体処理液に添加し10秒間撹拌後，Xpert *C. difficile* カートリッジの検体注入口に分注し，測定開始後45分で自動的に結果が得られる。ゴールドスタンダードと比較し，唯一94%の感度を得た[10] *C. difficile* 検査のリアルタイムな検出によって，アウトブレイクの徴候を早期に察知し，伝播阻止のために環境の消毒法の変更やフルオロキノロン系の制限など，感染制御体制を講じることが可能となる。また，アウトブレイクが発生した場合は，感染制御介入のために有効なエビデンスを提供することになり，感染対策上，貢献度が高い検査として大いに期待できる。

II. BDマックス™
（日本ベクトン・ディッキンソン社）

A. 概　　要

BDマックス™は日本ベクトン・ディッキンソン(株)から発売された全自動核酸抽出増幅検査システムである。従来の核酸増幅検査は核酸抽出と増幅や検出の工程が別々になっているため，試薬調製・各工程間で溶液の入れ替え・別々の装置のセッティングなど，コンタミネーションや人為的ミスの発生するリスクが存在する。また，その作業は煩雑であり専門的知識も必要となる。対して，BDマックス™は検体から直接，核酸抽出～増幅～検出の工程を全自動で実施するため，従来法と比較して簡便・迅速に検査を実施することが可能であり，検査業務の効率化や用手法工程を削減し，統一された方法によって検査実施者によらず一定の精度を保った検査が実施できるといった利点がある。

B. システム

実際の操作は，①検体を準備，②Sample Tubeをラックにセット，③試薬をラックにセット，④PCR用カートリッジをセット，⑤ランスタート，という簡便なワークフローになっており，最大24検体の処理が可能である(**Fig. 3**)。

核酸は磁性粒子法による抽出で，血清・血漿・スワブ，CSF，尿などの検体種に対応したキットがある。核酸の増幅と検出はリアルタイムPCR法で実施し，5波長搭載のためマルチプレックス検出にも対応可能である。

試薬をセットする消耗品はURS(Utilized Reagent Strip)というストリップで，分注作業を行うためのチップや抽出試薬などを備えており，この中で核酸抽出～PCR反応溶液の調整までが実施される。分注作業時はピペッティングヘッドがストリップ間を横に跨がないように動作することにより，コンタミネーションのリスクを防いでいる。

調製されたPCR反応溶液は自動的に専用のカートリッジに充填される。カートリッジはマイクロ流路系のシステムとなっており，細い流路を通ってウェル内にPCR反応溶液が移行する。溶液が移行した後は流路が塞がれる仕組みになっており，反応終了後にPCR産物が外部に漏れることがないよう工夫されている。

使用する消耗品類はバーコードによって管理される。作業開始前にセットされているものが正しいか否かを装置が確認するため，人為的なミスが極力発生しないように装置を使用できる。

C. オープン試薬

昨今，全自動の装置は他社からも発売されているが，BDマックス™の大きな特長の一つにオープン

試薬を用いた測定がある。これは，実施者自身で核酸増幅を行うための試薬を用意すれば独自に測定系を構築できるものである。具体的には，BD マックス™用の核酸抽出キット，リアルタイム PCR 反応用試薬，目的の病原体を検出するためのプライマーやプローブを用意する。リアルタイム PCR 反応用試薬は他社製のものでも使用することは可能だが，BD マックス™専用のものも発売されており，こちらは内部コントロール検出用のプライマー等を含んでいるため便利である。

オープン試薬を利用すれば，希望する病原体や遺伝子の検出キットがメーカーから発売されていない場合でも独自に検出系を構築して運用できる可能性が考えられる。我々の施設では，VRE（バンコマイシン耐性腸球菌）の *van* 遺伝子を検出する系を構築し，in house 検査での利用を検討している。

D. 核酸増幅法によるアクティブサーベイランス

MRSA は分離される耐性菌の中でも多くの割合を占め，院内感染が問題となっている。入院患者や感染のリスクが高い ICU などで積極的に感染者や保菌者を発見し，感染対策に役立てようというアクティブサーベイランスを試みている施設もあり，その効果が期待されている。

MRSA の検出には培養法が用いられているが，結果が判明するまで約 1 日掛かるため，迅速性に欠ける。一方，核酸増幅法を用いた MRSA 検出用キットも市販されており，この方法は培養することなく検体から直接実施できるため約 2 時間で結果が得られる。Izumikawa ら[11]は，呼吸器病棟入院患者における MRSA アクティブサーベイランスを実施し，培養法と比較して核酸増幅法が迅速・高感度・高特異度に結果が得られ，核酸増幅法が有用であったと報告している。また，Taguchi らの報告[13]では，救命センターなど院内伝播リスクの高い箇所においてMRSA アクティブサーベイランスを実施することにより，感染予防に有用であると報告している。その他にも，アクティブサーベイランスに核酸増幅法を用いた事例が報告されている[13][14]。

E. 今　後

現在発売中の測定キットは MRSA 検出用であるが，その他には腸内細菌，耐性遺伝子，呼吸器関連の病原体検出を対象とした測定キットの発売も予定されている。全自動核酸抽出増幅検査システムである BD マックス™による簡便で迅速な核酸抽出・増幅検査を導入することで，院内での病原体早期発見による感染症予防・院内伝播の予防など，様々な効果が今後期待される。

III. Anyplex™ II シリーズ（Seegene 社）

A. 概　要

Anyplex II は，Seegene 社（韓国）が特許を持つ DPO™（Dual-Priming Oligonucleotides）という非常に特異性の高いプライマーと，TOCE™（Tagging Oligonucleotide Cleavage and Extension）というマルチプレックス技術を組み合わせることにより，1 チューブで 10 種類以上の項目をリアルタイム PCR 機器において同時に測定することが可能となっている。

B. DPO と TOCE について

DPO は，PCR プライマーに関する PCR 関連技術である。DPO は安定的に標的核酸に結合する 5' 末端領域と数塩基しか結合しない不安定な 3' 末端を持つプライマーで，それら 2 領域の間にポリイノシン酸リンカーを付加することで，不安定性が増し，完全に配列が一致した場合のみ増幅が可能となる。この DPO プライマーを用いることで，10 種類以上のマルチプレックス PCR が可能となる。TOCE とは，Tagging Oligonucleotide Cleavage and Extension の頭文字を取ったもので，リアルタイム PCR のプラットフォームで，マルチプレックスを可能とした技術である（**Fig. 4**）。TOCE は以下のステップで行われる。まず，材料として標的 1 項目につき，DPO フォワードプライマーと DPO リバースプライマー，Pitcher プローブ，Catcher プローブの 4 種類のオリゴ DNA が必要となる。PCR の過程で，Forward プライマーと Reverse プライマー，Pitcher プローブが標的核酸に結合し，DNA ポリメラーゼにより伸張反応が起こる。その際，結合した Pitcher プローブが DNA ポリメラーゼのヌクレアーゼ活性により切断され，Pitcher プローブの 5' 末端のタグ配列が遊離する。遊離したタグ配列が，1 本鎖の Catcher プローブの 3' 末端領域と結合し，DNA ポリメラーゼにより伸張反応が起き，二本鎖 DNA となる。Catcher プローブには，蛍光物質と消光物質が標識されており，通常一本鎖のときは，高次構造をとり，蛍光物質が消光された状態になっている。しかし，Pitcher プローブのタグ配列が結合し，二本鎖を形成することによって Catcher プローブが蛍光を発する。この蛍光をリアルタイムに測定することによっ

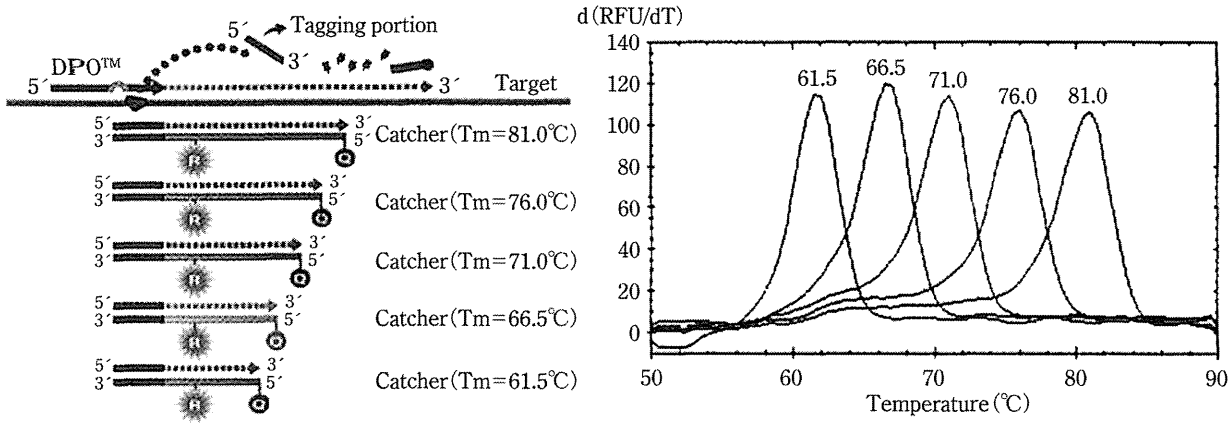

Figure 4　TOCE™(Tagging Oligonucleotide Cleavage and Extension) technology.
One unique feature of TOCH™ is the "Catcher", which is a fluorescently labeled artificial template that generates the signal for each target amplification. The Catcher melting temperature (Catcher-Tm) can be controlled by adjusting the sequence and length of the Catcher. For TOCE™ assay optimization, the Catcher-Tm can be easily adjusted and is not limited by the target sequence.

て，リアルタイム PCR が可能となる。また，最終産物の二本鎖 Catcher プローブに熱をかけて融解曲線解析を行うと，Catcher プローブの長さによってある温度に融解のピークを呈する。項目によってそれぞれ長さの異なる Catcher プローブを用意することで，一蛍光あたり 4～6 種類までの項目が検出可能となる。一般のリアルタイム PCR 機器であれば，4 種類以上蛍光物質を検知できることから，1 チューブで 10～20 項目が同時に測定可能となり，検体の分注の手間を大きく省くことができる。測定の流れとしては，検体から核酸を抽出した後，リアルタイム PCR 機器(Anyplex II シリーズは Bio-rad 社製 CFX96 を推奨)に抽出核酸と試薬の混合液をセットし，50 サイクルの PCR を行った後，55 から 85 度まで徐々に温度を上げ，融解測定を行う。その後，専用解析ソフトによって，標的の有無を定性的に判別する。また，Seegene 社が販売している自動核酸抽出・試薬調製器である Nimbus 4-probe を用いることにより半自動化も可能となっている。この DPO と TOCE を用いた Anyplex II のラインナップは，現在 5 種類が韓国，ヨーロッパで承認を得ている。日本においては，研究用として使用可能であるが，今後診断薬として開発される可能性がある。

C. Anyplex II について

Anyplex II の 5 種類は，すべて感染症関連試薬となっている。1 つ目は Anyplex™ II MTB/MDR/XDR で，結核菌関連検出試薬で，リファンピシンおよびイソニアジドの耐性を検出できる多剤耐性結核菌(MDR)と注射剤，フルオロキノロン耐性の超多剤耐性結核菌(XDR)を検出することが可能である。2 本のチューブでそれぞれ，MDR，XDR を同定する。2 つ目はヒトパピローマウイルス(HPV)の子宮頸がんに対するハイリスク型 19 種類(16, 18, 26, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 66, 68, 69, 73, 82)とローリスク型 9 種(6, 11, 40, 42, 43, 44, 54, 61, 70)の計 28 種類のタイピングキット(Anyplex™ II HPV28 Detection)であり，現在日本で承認されているハイリスク型 13 種類(もしくは 14 種類)よりも多くのハイリスク型を検出できる。3 つ目は尿道炎などの性感染症原因微生物 7 種類同時検出試薬(Anyplex™ II STI-7 Detection)である。*Chlamydia trachomatis*，*Neisseria gonorrhoeae*，*Mycoplasma genitalium*，*Mycoplasma hominis*，*Ureaplasma urealyticum*，*Ureaplasma parvum*，*Trichomonas vaginalis* を 1 チューブで検出することが可能であり，日本において既承認項目であるクラミジア，淋菌が含まれる。4 つ目(Anyplex™ II RB5 Detection)は，培養が困難もしくは培養に時間のかかる呼吸器感染症原因菌 5 種同時検出キットとなっている。対象項目は，一般的に非定型肺炎と言われる肺炎マイコプラズマや肺炎クラミジア，レジオネラ菌，百日咳菌，パラ百日咳菌の 5 菌種である。最後の 5 つ目の製品は，呼吸器感染症関連ウイルス検出キット(Anyplex™ II RV16 Detection)であり，型判別を含め 16 種類のウイルスを 2 本のチューブで

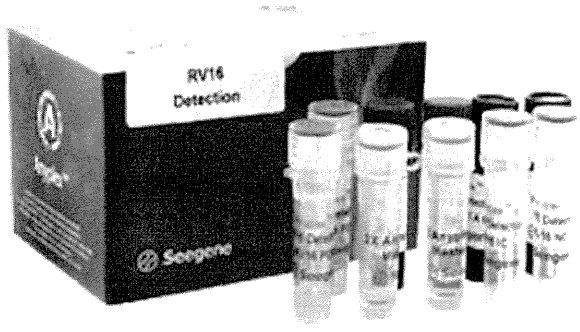

Figure 5 Anyplex™ II RV16 Detection(呼吸器関連ウイルス検査)キット.

Anyplex™ II RV16 Detection detects 16 respiratory viruses (adenovirus, influenza A virus, influenza B virus, parainfluenza virus 1, parainfluenza virus 2, parainfluenza virus 3, parainfluenza virus 4, rhinovirus A/B/C, RS virus A, RS virus B, bocavirus, coronavirus 229R, coronavirus NL63, oronavirus OC43, metapneumovirus, and enterovirus) in a single real-time PCR.

検出できる(**Fig. 5**)。16種類のウイルスは，アデノウイルス，A型・B型インフルエンザ，1・2・3・4型パラインフルエンザ，ライノウイルス，A型・B型RSウイルス，ボカウイルス，メタニューモウイルス，コロナウイルス229E株，NL63株，OC43株，エンテロウイルスが検出可能である。

ここで，性感染症キットを用いた臨床データによると，韓国で採取された検体897例について，Anyplex IIキットと他社キットを比較した結果，*C. trachomatis*については，感度，特異度が100%であった[15]。*N. gonorrhoeae*の感度に関しては，BD ProbeTec™(SDA法)が約80%であったのに対し，本キットは100%を示した。*M. genitalium*に関しては，AmpliSens®と比較した結果，ほぼ同じ検出感度，特異度であった。他の4種の菌に対しても，他のキットと比較し，同等もしくは優れた結果を示した。本論文よりAnyplex IIはマルチプレックスでありながら，感度，特異度も高く，非常に有用性の高い検出試薬であることが示されている。

D. 今　後

日本における感染症原因菌同定用の体外診断薬は，多項目検出試薬はほとんどなく*C. trachomatis*と*N. gonorrhoeae*の2項目同時測定のみである。これらはもともと個別測定であったが2菌種同時測定することにより検体量だけでなく薬価も抑えられた。感染症領域においては今後も少ない検体量で多くの情報(原因菌同定・薬剤耐性等治療にも反映できる確度の高い診断)を得ることができるマルチプレックス化への要望は増加すると考えられるが，臨床試験の難しさ，薬事承認や保険点数の設定など未知の課題が山積している。

IV. パンサー™システム(ホロジック社)

A. 概　要

2012年ホロジック社とGen-Probe社との経営統合により，日本におけるアプティマ®(TMA法)試薬ならびに遺伝子解析装置パンサー™システムの製造販売元が富士レビオ社よりホロジックジャパン社へ承継された。現在ホロジック社が新しく販売するパンサー™システムは，「生化学・免疫検査分野での装置と同様の自動化を遺伝子検査室で可能とする」というコンセプトで開発された，次世代型遺伝子解析装置である。

本システムは検査室へ検体容器の到着後，検体ラックに専用の容器をセットし，装置にラックを装填するだけで，核酸の抽出・増幅・検出・結果報告までの一連の工程，ならびに核酸増幅反応による核酸増幅産物の不活発化処理までを全自動で行い，オペレーターの作業時間を大幅に削減することができる。また，「Random Access Sampling」機能を搭載し，同一のラック内で異なった検査項目をランダムに測定できるだけでなく，検体ラックの投入が一日中連続的に可能なため，随時検査が可能であり，従来のようにバッチでの検体処理に要する手間・無駄を省き，最適な検査ワークフローを実現する事が可能である。付属する機器類が必要なく，コンパクトに集約された一体型デザインを特徴としており，ラボにおける設置スペースの有効活用も可能となる。

B. キ ッ ト

パンサー™システムの専用検査キットとして，現在国内ではアプティマCombo 2クラミジア／ゴノレア(以下AC2)がホロジック社より販売されている。本キットは2006年6月に本邦で承認され，CTおよびNGを同一の試験管内で同時にかつ単独でも測定が可能であることを特徴としている。AC2はDNAよりも1つの細胞に多く含まれるrRNAをターゲットとしており，微量な細胞数しか採取できなかった検体においても，見逃しがなく高感度での検出が期待される。

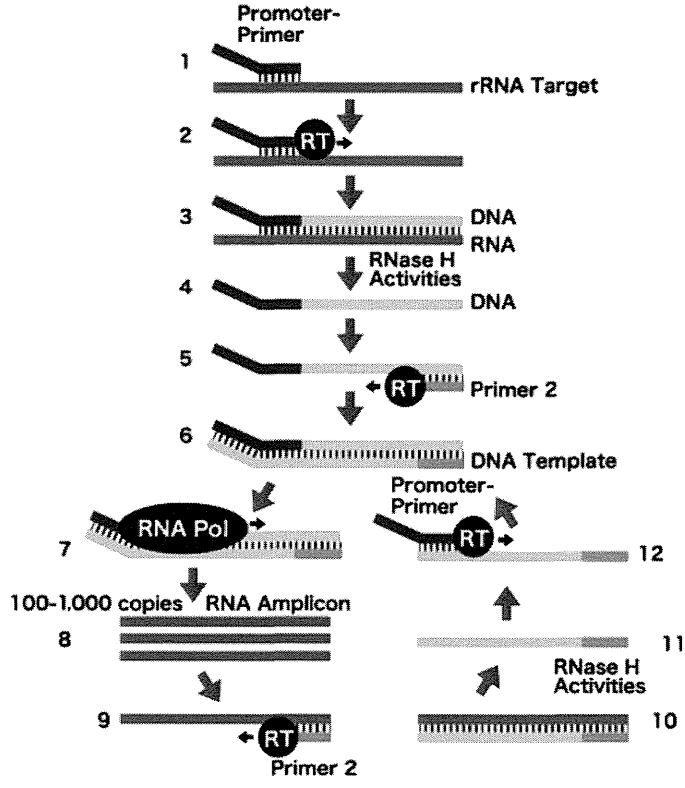

Figure 6　Transcription-Mediated Amplification（TMA）法.

- ステップ 1～3: プロモーター領域が付加されたプライマーが，標的 RNA の相補的な部分にハイブリダイズし，逆転写酵素（RT）により相補 DNA が合成される。
- ステップ 4: 前のステップで得られた RNA：DNA ハイブリッドの RNA 鎖が，逆転写酵素の RNase H 活性により解される。
- ステップ 5～6: 第 2 のプライマーが，DNA 鎖にハイブリダイズし，逆転写酵素によりこの DNA 鎖に相補的な DNA 鎖が合成され，二本鎖 DNA となる。
- ステップ 7: RNA ポリメラーゼ（RNA Pol）が，二本鎖 DNA 内のプロモーター配列を認識し，転写を開始する（DNA から RNA が合成される）。
- ステップ 8～12: 新たに合成された RNA アンプリコンは，それぞれ TMA サイクルに再導入され，指数的数の RNA アンプリコンが合成される。

C. TC 法と TMA 法

検体の前処理法は Target Capture 法（TC 法）を採用している。TC 法は標的核酸を特異的なプローブを用いて捕獲し，磁性微粒子上に吸着させる。この磁性微粒子を磁石で集めた後溶液を吸引除去することにより標的核酸のみを単離することができる。そのため，検体中の血液や尿中成分，非特異核酸分子，蛋白などを洗浄除去でき，特異性が向上するため，反応の阻害が大幅に低減できる。

核酸増幅法は Transcription Mediated Amplification（TMA）法を採用している。TMA 法は 2 種類の酵素を用いた核酸増幅法である。まず，1 ペアのプライマー，逆転写酵素により標的 1 本鎖 RNA から 2 本鎖 DNA を合成する。プライマーの片方にはプロモーター領域が存在しており，それを鋳型として T7RNA ポリメラーゼにより標的 RNA を増幅する（**Fig. 6**）。

AC2 の基礎的な外部評価データとして，精製したクラミジア基本小体（EB）を段階希釈したものを試料とした AC2 の感度データでは，AC2 は 0.005 IFU/mL という非常に感度に優れた結果が確認されている（**Table 1**）[16]。加えて，阻害物質の影響についても，リン酸塩と Fe イオンを添加した試験検体を用い検討した結果，アプティマ Combo 2 はこれらの物質による反応阻害を受けないことが示された[16]。また検

Table 1　Combo 2 クラミジア/ゴノレア

Elementary Body (EB) Suspension		アプティマ™ Combo2 クラミジア/ゴノレア	
Inclusion Forming Units	Equivalent EBs	Mean total	Results
mL-1		Relative Light Units	+
50	200	1,217	+
5	20	1,111	+
0.5	2	1,062	+
0.05	0.2	878	+
0.005	0.02	288	+
0.0005	0.002	12	−
0.00005	0.0002	14	−
0.000005	0.00002	13	−

出用プローブに，特異性の高い配列を選択したことにより，特異度が高く，特に口腔常在菌ナイセリア属による交差反応が極めて少ないというデータも示されている[17]。AC2 は尿・子宮頸管擦過物・尿道擦過物に加え，咽頭擦過検体においても検体適用となっており，咽頭検体での核酸増幅法の検査において，国内では日本性感染症学会ガイドライン 2011 で推奨を受けている[18]。

V. 結　　語

質の高い効率的な医療を提供するために，迅速な診断と迅速な治療開始に寄与する臨床検査が求められる。遺伝子検査は迅速診断・確定診断のうえで，検査室において，診療に直結した非常に重要な検査の位置づけとなる。しかしながら，現状の検査室において遺伝子検査を実施するとなると，習熟度のある検査スタッフの確保が必要となり，検体の前処理から核酸の抽出・増幅・検出までの作業の手間がかかる。また，検査項目ごとの検体数の少なさや測定する検査項目ごとに各メーカーの分析装置が異なるため，それぞれの装置への初期投資にかかるコストや，機器の設置スペースの確保など多様な観点から克服すべき問題が多く，医療機関内で遺伝子検査を実施するためには簡便性・自動化・費用対効果などが今後の課題である。さらに，一度に多数の検査が同時にできることは遺伝子検査の特徴でもあるが，臨床現場で保険点数をどうするかなど行政側にも解決すべき課題が残されている。

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Adjuvant effect of Japanese herbal medicines on the mucosal type 1 immune responses to human papillomavirus (HPV) E7 in mice immunized orally with *Lactobacillus*-based therapeutic HPV vaccine in a synergistic manner

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ABSTRACT

The Japanese herbal medicines, Juzen-taiho-to (JTT) and Hochu-ekki-to (HET), have been shown to enhance humoral immune responses to vaccine antigen when used as adjuvants for prophylactic vaccines. However, their adjuvant effect on mucosal cellular immune responses remains unstudied. The precursor lesion of cervical cancer, high-grade CIN that expresses HPV E7 oncoprotein ubiquitously is a target for HPV therapeutic vaccines that elicit mucosal E7-specific type 1 T cell responses. We have demonstrated that oral immunization with recombinant *Lactobacillus casei* expressing HPV16 E7 (LacE7) is more effective in eliciting mucosal E7-specific IFNγ-producing cells than subcutaneous or intramuscular antigen delivery. Here we report the synergistic effect of an oral *Lactobacillus*-based vaccine and Japanese herbal medicines on mucosal immune responses. Oral immunization of mice with LacE7 plus either a Japanese herbal medicine (JTT or HET) or a mucosal adjuvant, heated-labile enterotoxin T subunit (LTB), promotes systemic E7-specific type 1 T cell responses but not mucosal responses. Administration of LacE7 plus either Japanese herbal medicine and LTB enhanced mucosal E7-specific type 1 T cell response to levels approximately 3-fold higher than those after administration of LacE7 alone. Furthermore, secretion of IFNγ and IL-2 into the intestinal lumen was observed after oral administration of LacE7 and was enhanced considerably by the addition of Japanese herbal medicines and LTB. Our data indicated that Japanese herbal medicines, in synergy with *Lactobacillus* and LTB, enhance the mucosal type 1 immune responses to orally immunized antigen. Japanese herbal medicines may be excellent adjuvants for oral *Lactobacillus*-based vaccines and oral immunization of LacE7, HET and LTB may have the potential to elicit extremely high E7-specific mucosal cytotoxic immune response to HPV-associated neoplastic lesions.

1. Introduction

Human papillomavirus (HPV) infection is a major risk factor for the development of cervical cancer which is the second most common cancer among women [1]. HPV prophylactic vaccines hold promise to reduce the worldwide incidence of cervical cancer. However, limitations in current HPV vaccine strategies make the development of HPV therapeutic vaccines for the treatment of HPV-associated lesions essential. HPV E7 is an attractive target protein for HPV therapeutic vaccine strategies that are directed against a precursor lesion of cervical cancer, high-grade cervical intraepithelial neoplasia (CIN) [2]. Many therapeutic vaccines against HPV E7 have been developed and several clinical vaccination trials against high-grade CIN have been completed [3–11]. However, no therapeutic HPV vaccines are yet available. The current vaccine candidates have been shown to elicit systemic cellular immunity after intramuscular or subcutaneous injection and clinical trials have shown cellular immune responses to the vaccines in peripheral monocytes but fail to show local immunity in the cervical mucosa after vaccination. Cervical mucosal lesions may be poorly responsive to systemic cellular immunity since precursor lesions develop in the mucosal epithelium; mucosal intraepithelial lymphocytes (IELs) should be the central effector cells for the elimination of CIN. Lymphocytes involved in the mucosal immune system are found in the inductive sites of organized mucosa-associated lymphoid tissues and in a variety of effector sites such as the mucosa of the intestine, respiratory tract and genital tract [12]. The efficient homing of lymphocytes to the gut is dependent on the homing receptors integrin α4β7 [13]. Several studies have demonstrated that gut-derived integrin $\alpha4\beta7^+$ lymphocytes subsequently home to the genital mucosa [14–17].

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We have reported previously that the oral *Lactobacillus*-based vaccine expressing HPV16 E7 (LacE7) has substantial potential to be a novel HPV therapeutic vaccine [18]. Oral immunization with LacE7 elicited E7-specific IFNγ-producing cells (T cells with E7-type1 immune responses) among integrin $\alpha4\beta7^+$ mucosal lymphocytes collected from gut mucosa. In our previous study, oral immunization with LacE7 preferentially elicited E7-specific type1 T cell responses in mucosal lymphocytes when compared to splenocytes. Taken together with the data that gut-derived integrin $\alpha4\beta7^+$ T cells home to the cervical mucosa [19], we predicted that vaccine-induced mucosal $CD4^+$ and $CD8^+$ T cells will have antitumor effects on mucosal HPV E7-related neoplastic lesions.

Traditional Chinese herbal medicines and their Japanese counterparts, Japanese herbal medicines, are used not merely to improve weak constitutions but also to suppress many constitutional symptoms. The Japanese herbal medicines, Juzen-taiho-to (JTT) and Hochu-ekki-to (HET), have been reported to exert beneficial effects on various aspects of the immune response [20] and are thought to have great potential as adjuvants for prophylactic vaccination against a variety of microbes [21–23]. JTT's immunomodulatory actions include an enhancement of the mitogenic activity of spleen cells, a promotion of phagocytosis and anti-tumor effect [24,25]. HET activates natural killer cells and macrophages [26,27]. Orally administered HET increases antibody titers against influenza virus in mice immunized with influenza vaccines and promotes secretory IgA production after oral OVA vaccination [28,29].

Viewing the actions of JTT and HET on innate immunity within the intestinal mucosa after oral vaccination, we hypothesized that concurrent oral administration of JTT or HET and LacE7 would enhance mucosal cellular immune responses against HPV16 E7. To address the immunomodulatory effects of JTT or HET on anti-E7 immune responses, mice were given oral JTT or HET in addition to a LacE7 oral vaccine with or without the known adjuvant, a heat-labile lymphotoxin T subunit (LTB).

2. Materials and methods

2.1. Immunization protocols

LacE7 was provided from BioLeaders Corp. (Korea) and GENOLAC BL Corp. (Japan). LacE7 was generated from the recombinant *Lactobacillus casei* expressing HPV16 mutated E7 as previously described [18] and attenuated using heat. The attenuated *L. casei* were purified by washing several times with distilled water then dried to powder. LacE7 was insoluble in water-based solvents. Six-week-old female SPF C57BL/6 mice (CLEA Japan Inc., Japan) were used for immunization experiments. 1.0 mg/head of LacE7 were administered four times at weeks 1, 2, 4, and 6. All inoculums were suspended in PBS (200 μL/head) and administered once per day for five days each week via an intra-gastric tube after 3 h of fasting.

The Japanese herbal medicines, JTT or HET (40 mg/head/day, gifted from Dr. Keiichi Koizumi, University of Toyama) were mixed with powdered foods (5 g/head/day) which were taken consumed completely by five mice in a single cage. JTT or HET was administered to mice every day during each of the four rounds of LacE7 administration (weeks 0–6). Heat-labile *Escherichia coli* lymphotoxin, B subunit (LTB: 10 μg/head) was added to each LacE7 inoculum and administrated orally on the third day of each round of vaccination.

2.2. Sample collection

Lymphocytes, serum and intestinal washes were collected from immunized mice one week after the last inoculation (at week 7). After sacrifice, intestine, spleen and peripheral blood were obtained from five mice. Spleens were washed 3 times in HBSS. For intestinal specimens, the inside of intestinal tract was washed with 10 mL of HBSS with protease inhibitors after feces removal. The collected sera and intestinal washes were stored at −80 °C until use.

2.3. Preparation of murine splenocytes and intestinal mucosal lymphocytes

The intestines were opened longitudinally and shaken vigorously in RPMI1640 containing 10% FBS, 100 units/mL of penicillin and 100 μg/mL of streptomycin for 30 min at 37 °C. The resulting cell suspensions were passed through a BD Falcon Cell-strainer (BD Bioscience, USA) to remove tissue debris and were subjected to discontinuous density gradient centrifugation in a 15 mL tube layered from the bottom with 70% and 40% Percoll PLUS (GE Heaithcare UK Ltd., England). The interface between the 70% and 40% layers contained lymphocytes with a cell viability of more than 95%. Splenocytes were prepared by gently teasing the spleen in PBS. Clumped debris was removed by centrifugation. Approximately $5–10 \times 10^6$ intestinal mucosal lymphocytes and 10^7 splenocytes were obtained from individual mice.

2.4. ELISPOT assays

50 μL of intestinal mucosal lymphocytes or splenocytes (5×10^6 cells/mL) were incubated for 24 h at 37 °C with antigen presenting cells comprised of 50 μL of splenocytes (5×10^6 cells/mL) treated with mitomycin C (75 μg/mL, Sigma, USA), and washed three times with PBS. 10 μL of synthetic peptide (working conc. = 1 μg/mL) corresponding to amino acids 49–57 of HPV16 E7 (a reported CTL epitope for C57BL/6 mice), mitogen (PMA 40 ng/mL + ionomycine 4 μg/mL), or medium alone (negative control) were added to a 96-well ELIIP plate (Millipore, USA) coated with anti-mouse IFNγ monoclonal antibodies from the Mouse IFNγ Kit (MABTECH AB, Sweden). IFNγ spot numbers were analyzed with a fully automated computer assisted video imaging analysis system, KS ELISPOT (Carl Zeiss Vision, Germany).

2.5. Cytokine measurements

Intestinal washes obtained from five mice were pooled and cytokine concentrations measured using the mouse Th1/Th2 ELISA Ready SET Go Kit (BD Bioscience, San Diego, CA, USA), which include IFNγ and IL-2 as representative Th1-type cytokines. The cytokine levels in each sample were normalized by total protein concentration. Measurements were repeated at least three times.

2.6. Statistical analysis

ELISPOT and ELISA data were presented as means ± standard deviations. Measurements and relative rates were compared between the immunization groups (5 mice/each group) using non-paired, two tailed Student's *t*-tests. A *p*-value of <0.05 was considered to be significant.

3. Results

3.1. The adjuvant effect of Japanese herbal medicines on E7-specific type 1 T cell responses

To examine the effect of oral administration of LacE7 vaccine plus Japanese herbal medicines on E7-specific type 1 T cell responses, the number of IFNγ-producing cells among mucosal lymphocytes or splenocytes was assessed by ELISPOT assay (Fig. 1). Each group of five mice was administered LacE7 (1.0 mg/head) orally or LacE7 plus JTT or HET (40 mg/head). JTT and HET were

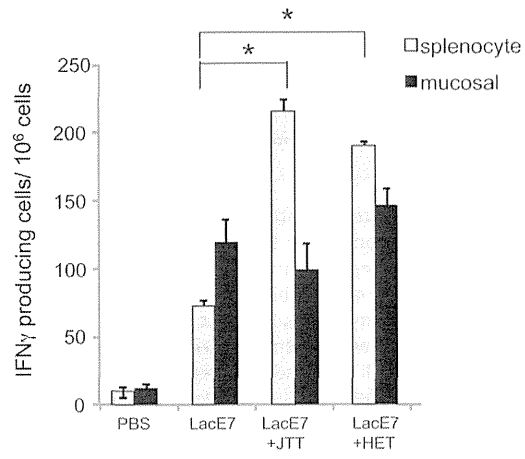

Fig. 1. Adjuvant effects of Japanese herbal medicines on type 1 T cell responses in mice orally immunized with Lac E7. The number of E7-specific IFNγ-producing cells among intestinal mucosal lymphocytes and splenocytes were assessed using ELISPOT assay. Five mice per group were immunized with LacE7 (1.0 mg/head) or PBS four times at weeks 1, 2, 4, and 6. JTT or HET was administered to mice every day during the four rounds of LacE7 administration. Mucosal lymphocyte and splenocytes were collected from immunized mice one week after last inoculation (at week 7) and approximately 10^5 of each type of lymphocyte were stimulated with the E7 peptide corresponding to HPV16E7 49–57 aa. Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

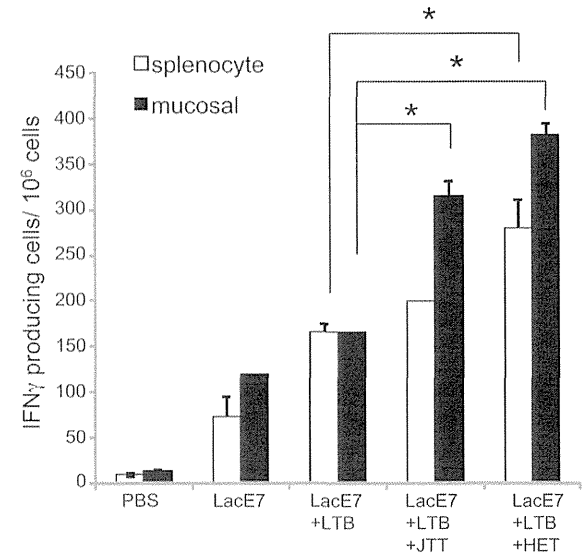

Fig. 2. Synergistic adjuvant effect of Japanese herbal medicines and LTB on type 1 T cell response. LTB (10 μg/head) was added to each LacE7 inoculum and administrated orally on the third day of each round of vaccination. This was performed in mice contemporaneously exposed to JTT, HET or control (no exposure). The number of E7-specific IFNγ producing cells among the collected intestinal mucosal lymphocytes and splenocytes was assessed using the ELISPOT assay as shown in Fig. 1. Mean values with standard deviations are presented. Asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

administered to mice as supplements to powdered food every day during four rounds of the LacE7 oral immunization. To detect potential adjuvant effects of the supplements on mucosal and systemic immunity, intestinal mucosal lymphocytes and splenocytes were collected from each mouse one week after the last immunization. The numbers of E7-specific IFNγ-producing cells among both mucosal lymphocytes and splenocytes increased significantly in LacE7-immunized mice but not in non-immunized (PBS) mice (Fig. 1). Oral immunization with LacE7 elicited a predominant mucosal E7-specific type 1 T cell response with E7-specific IFNγ-producing cell levels approximately 1.5–2.0-fold higher than those among splenocytes. Administration of LacE7 plus JTT or HET significantly improved systemic E7-specific type 1 T cell responses in splenocytes. However, neither JTT nor HET exhibited significant adjuvant effects on mucosal type 1 T cell responses (Fig. 1).

3.2. *Adjuvant effects of the Japanese herbal medicines when combined with LTB on mucosal immune responses*

Our initial data suggested that the use of additional adjuvants might be necessary to improve the mucosal cellular immune response to E7. We therefore repeated our investigations, adding oral LTB to LacE7 with each round of LacE7 oral immunization. Although the levels of E7-specific type 1 T cell response in mice given LacE7 plus LTB tended to increase, no significant differences were noted when comparing LacE7/LTB to LacE7 alone (Fig. 2). Mice exposed to either JTT or HET together with LTB and-LacE7 had improved mucosal E7-specific type 1 T cell response with approximately 2–2.5-fold higher levels of E7-specific mucosal IFNγ-producing cells when compared with sole exposure to LacE7 plus LTB (Fig. 2). Comparing Figs. 1 and 2, we noted that the addition of LTB to LacE7 plus either JTT or HET doubled the number of the IFNγ-producing cells among mucosal T cells, but not splenocytes. These data indicated that LTB and the Japanese herbal medicines act synergistically on the mucosal type 1 T cell response elicited by LacE7.

3.3. *Local cytokine production induced by oral immunization with LacE7, LTB and Japanese herbal medicines*

To confirm the characteristics of local cellular T cell responses stimulated by oral immunization, type 1 cytokine secretions were measured in the mucosal compartment. Levels of IFNγ and IL-2 production in intestinal washes obtained from immunized mice were measured by ELISA (Figs. 3 and 4). Both IFNγ and IL-2 levels in the mucosal fluid increased significantly in mice immunized orally with LacE7 when compared with non-immunized mice (PBS), consistent with a previous data that mucosal administration of *L. casei* alone induces Th1 cytokine production in a mucosal compartment [30]. Using comparisons mimicking those in Fig. 2, LacE7 plus either JTT or HET and LTB promoted secretion of both IFNγ and IL-2 into the intestinal lumen (Figs. 3 and 4). The secretion levels were 6–8-fold higher for IFNγ (Fig. 3) and 2–4-fold higher for IL-2 (Fig. 4) when compared with LacE7 alone. Administration of LacE7 plus LTB did stimulate increased cytokine secretion when compared with LacE7 alone. These results confirm that JTT or HET have synergistic effects when added to LacE7/LTB oral immunization protocols on local Th1 cytokine secretion, as well as the induction of E7-specific IFNγ-producing cells.

4. Discussion

The therapeutic HPV vaccines tested to date can induce enhanced cellular immune responses but none have demonstrated clinical efficacy against CIN [31–33]. We hypothesize that by using intramuscular or subcutaneous injection strategies, these approaches promote systemic cellular immunity, but not local mucosal immunity. Intraepithelial lymphocytes (IELs) residing in the cervical mucosa are most likely to represent the central effector cells for elimination of CIN and systemic vaccination with HPV E7 is not thought to elicit and retain enough E7-specific CTL within the cervical mucosa to eliminate CIN. We have previously observed

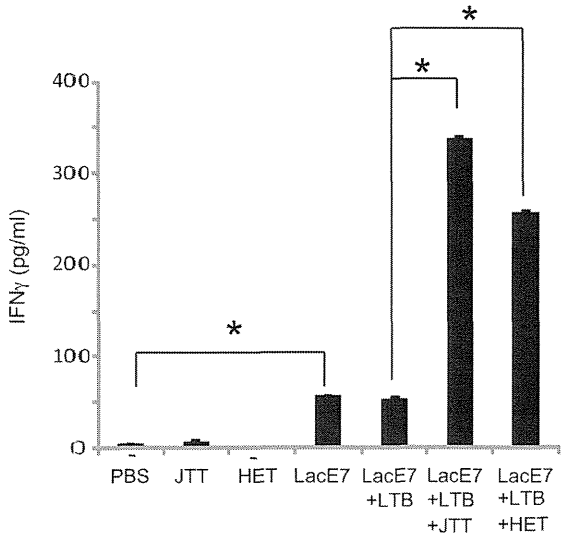

Fig. 3. IFNγ secretion into the intestinal compartment after immunization with LacE7 plus JTT or HET and LTB. IFNγ levels in the intestinal washes were measured by ELISA. The intestinal washes were collected at the same time points that were assessed in Fig. 1. Cytokine levels in each sample were normalized to corresponding total protein concentrations. Mean values with standard deviations are presented. The asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

and reported the induction of integrin $\alpha 4\beta 7^+$ mucosal T cells that provide E7-specific type 1 T cell responses after oral administration of LacE7 to mice [18]. We have also demonstrated that 25–30% of the $CD3^+$ cervical lymphocytes are integrin $\beta 7^+$ T cells [34]. In

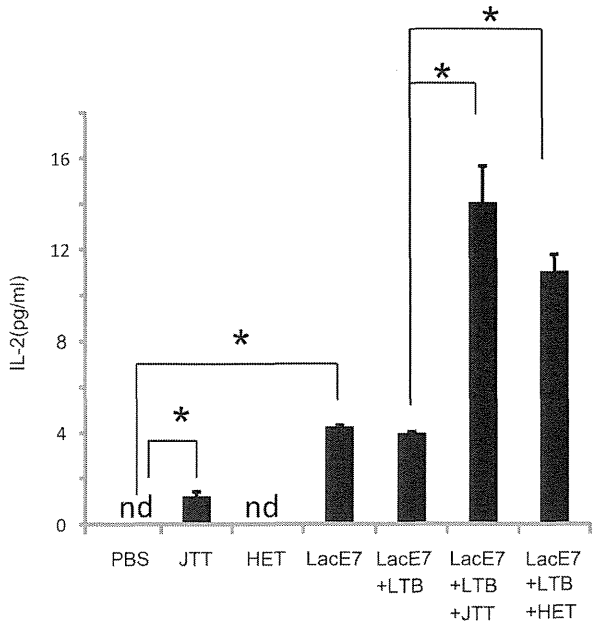

Fig. 4. IL-2 secretion into the intestinal compartment after immunization with LacE7 plus Japanese herbal medicine and LTB. IL-2 levels in the intestinal washes were measured by ELISA. The intestinal washes were collected at the same time points that were assessed in Fig. 1. Cytokine levels in each sample were normalized to corresponding total protein concentrations. Mean values with standard deviations are presented. The asterisks indicate those comparisons with statistical significance ($p < 0.05$) ($n = 5$).

our previous data, the number of vaccine induced E7-specific type1 T cells peaked at exposure levels of 1.0 mg/head and decreased with doses over 3.0 mg/head when mice were orally immunized with various doses of LacE7 (0.3–100 mg/head). We believe that 1.0 mg/head may be the optimal dose of LacE7 for induction of mucosal E7-specific type 1 T cells, because high-dose antigen may induce development of E7-specific regulatory T cells. These limitations led us to consider that the addition of an effective adjuvant agent might be more effective in improving E7-specific Th1 type responses than dose-escalation of LacE7. We chose to focus on two Japanese herbal medicines that have been reported to exhibit immunomodulatory effects.

Our data indicate that while JTT or HET alone exerts adjuvant effects on systemic but not mucosal type 1 T cell responses to LacE7, a combination of the mucosal adjuvant (LTB) with either Japanese herbal medicine dramatically improved the desired mucosal E7-specific type 1 T cell responses. These Japanese herbal medicine, when added to a conventional mucosal adjuvant, such as LTB, appear to act synergistically on mucosal vaccine-induced immune responses. The demonstrated adjuvant effects on mucosal immune response may be partially attributed to the strategy involving oral immunization of *L. casei*, which acts as an efficient vaccine carrier that delivers antigen across the gut to GALT but also exhibits its own vaccine adjuvant activities that promote type 1 T cell responses [4,35]. *Lactobacillus* species promote this type 1 T cell response polarization through interactions with dendritic cells (DCs) [36]. *Lactobacillus* activate DCs through TLR-2 and the activated DCs stimulate the proliferation of autologous $CD4^+$ and $CD8^+$ T cells and their secretion of IFNγ [37]. Recombinant *L. casei* alone can induce IFNγ production at mucosal sites [35]. Taken together, *L. casei* appears to be an excellent antigen delivery vehicle when mucosal type 1 T cell responses to vaccine antigen are desired. In our study, the levels of type 1 T cell responses to E7 barely increased in mice immunized with LacE7 and LTB when compared with LacE7 alone. However, the addition of Japanese herbal medicines to LacE7 and LTB resulted in two to three-fold higher levels of type 1 mucosal T cell responses when compared to LacE7 and LTB. In summary, the Japanese herbal medicines, JTT and HET act in synergy with *L. casei* and LTB in mucosal antigen delivery strategies. When Th1-type local T cell responses to vaccine antigen are desired, the combination of a Japanese herbal medicine and LTB promote efficient and mucosa-specific adjuvant activities when added to Lactobacillus delivery systems.

More specifically, the addition of specifically, the addition of specific Japanese herbal medicines and mucosal adjuvant to LacE7 may be an outstanding approach to generate E7-specific mucosal cytotoxic immune responses to HPV-associated neoplastic lesions.

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The Prevalence of Cervical Regulatory T Cells in HPV-Related Cervical Intraepithelial Neoplasia (CIN) Correlates Inversely with Spontaneous Regression of CIN

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Problem
Local adaptive cervical regulatory T cells (Tregs) are the most likely direct suppressors of the immune eradication of cervical intraepithelial lesion (CIN). PD-1 expression on T cells induces Tregs. No studies have quantitatively analyzed the Tregs and PD-1+ cells residing in CIN lesions.

Method of study
Cervical lymphocytes were collected using cytobrushes from CIN patients and analyzed by FACS analysis. Comparisons were made between populations of cervical Tregs and PD-1+ CD4+ T cells in CIN regressors and non-regressors.

Results
A median of 11% of cervical CD4+ T cells were Tregs, while a median of 30% were PD-1+ cells. The proportions of cervical CD4+ T cells that were Tregs and/or PD-1+ cells were significantly lower in CIN regressors when compared with non-regressors.

Conclusions
The prevalence of cervical tolerogenic T cells correlates inversely with spontaneous regression of CIN. Cervical Tregs may play an important role in HPV-related neoplastic immunoevasion.

Introduction

HPV infection is a major cause of cervical cancer and its precursor lesion, cervical intraepithelial neoplasia (CIN). Natural history studies of CIN[1,2] show that most infections and most CIN lesions resolve spontaneously; only a minority persists and progress to cervical cancer. Studies showing that HIV-infected women and patients who are under treatment with immunosuppressive agents have an increased incidence of CIN lesions[3,4] suggest that cell-mediated immune response against HPV viral protein is important in the control of HPV infection and progression to CIN. We have previously reported that the presence of gut-derived effector lymphocytes within the cervix plays an important role in local cell-mediated

immune responses and correlates with CIN regression.[5] The presence of robust local tolerogenic cervical T-cell responses to HPV-related neoplastic lesions would be predicted to attenuate the effects of these local effector responses. We hypothesized that the proportion of tolerogenic lymphocytes among the CD4+ T cells in the cervix would decrease among women experiencing CIN regression, thereby allowing full effect of the changes previously seen among local effector cells.

It has been reported that CD4+CD25+Foxp3+ regulatory T cells (Tregs) play an important role in tumor-associated immunoevasion in cancers (ovarian, uterine cervical, endometrial, lung, breast, pancreas, renal cell, and thyroid cancers) as well as in other proliferative disorders such as melanoma and hepatoma.[6–15] Mechanisms underlying Treg suppressive functions have been abundantly reported. The high expression of CD25 (IL-2R) on Tregs has been thought to result in cytokine deprivation-induced apoptosis of effector T cells.[16] IL-10, TGF-β, and IL-35 are also important mediators of Treg suppressive function.[16] Tregs have been reported to suppress T effectors by ligating T-effector-expressed CD80, thereby inhibiting T-cell proliferation and cytokine production. Tregs kill effector T cells, other antigen-presenting cells, and NK cells in a manner dependent on granzyme and perforin.[16]

Natural Treg cells (nTregs) differentiate in the thymus and migrate to peripheral tissues while adaptive/induced Treg cells (iTregs) differentiate in secondary lymphoid organs and tissues including mucosa-associated lymphoid tissues (MALT).[17] iTregs play essential roles in mucosal tolerance, in the control of severe chronic allergic inflammation, in the prevention of parasite and other microorganism clearance, and in the obstruction of tumor immunosurveillance while nTregs have roles in preventing autoimmunity and preventing exaggerated immune responses. iTregs appear in the mesenteric lymph nodes during induction of oral tolerance, differentiate in the lamina propria of the gut in response to microbial signals, and are generated in chronically inflamed tissues. At a minimum, Foxp3+ iTreg development requires TCR stimulation and the cytokines TGF-β and IL-2. Integrin αEβ7+ dendritic cells (DCs) residing in the MALT produce both TGF-β and retinoic acid (RA), which mediate the differentiation of naïve T cells into Foxp3+ iTregs.[17]

The programmed cell death-1 (PD-1) and PD-ligand (PD-L) pathway is also critical in the suppression of immune responses. PD-1 is a molecule inducibly expressed on peripheral CD4+ and CD8+ T cells, NKT cells, B cells, monocytes, and some DC subsets when these cells are activated by antigen receptor signaling and cytokines.[16] nTregs and iTregs can express PD-1 and PD-L1, and the expression of ligand and receptor on the same cell conveys interesting implications. Engagement of PD-1 by its ligands during T-cell receptor (TCR) signaling results in two possible T-cell responses: 1) a diminution in T-effector responses and 2) an augmentation in differentiation of naïve T cells into Foxp3+ iTreg in a TGF-β-dependent manner.[16] There are synergistic effects between the PD-1/PD-L1 pathway and TGF-β in promoting Treg development. PD-L1 is expressed on a wide variety of tumors, and high levels of PD-L1 expression strongly correlate with unfavorable prognosis in a number of cancers.[18] To this point, ligation of PD-1 may induce and maintain iTregs within the tumor microenvironment, enhance the suppression of anti-tumor T-cell responses, and thereby allow tumor progression.

Several previous studies have shown that the prevalence of Tregs among PBMCs increases in CIN patients when compared with healthy controls.[19,20] These studies assess populations of circulating Tregs using flow cytometry. Characterization of the local lymphocytes residing in cervical lesions should better reflect local immune responses to pathogen. While Nakamura et al.[21] used Foxp3 immunostaining of human CIN lesions to report the number of local Foxp3+ cells residing in the CIN lesions by immunostaining of the tissues for Foxp3 and report that the number of Foxp3-immunoreactive cells is higher in CIN3 lesions than normal or CIN1-2 lesions, no studies have quantitatively assessed populations of local Tregs, likely iTregs, in the CIN lesions using flow cytometry. Possible associations between iTregs and the natural course of CIN have also never been studied.

We have previously characterized cervical lymphocytes collected from CIN lesions using a cytobrush and have demonstrated that the majority of cervical lymphocytes in these lesions are CD3+ T cells (median 74%) and that half of the cervical CD3 + T cells are CD4+ (median 54%).[5] In the present investigations, we have analyzed the relative proportions of two tolerogenic T-cell subsets, CD25+Foxp3+ Tregs and PD-1+ T cells, among cervical CD4+ T cells collected from CIN lesions. To determine whether there was a correlation between the frequency of cervical tolerogenic T cell and the natural course of

CIN, comparisons were made between tolerogenic T-cell subsets in the lesions of CIN regressors and non-regressors.

Materials and methods

Study Population

Cervical cell samples were collected using a cytobrush from 24 patients under observation after being diagnosed with CIN by colposcopically directed biopsy. All women gave written informed consent, and the Research Ethics Committee of the University of Tokyo approved all aspects of the study. Patients with known, symptomatic or macroscopically visible vaginal inflammation, or sexually transmitted infections were excluded from our study. To study the association between cervical tolerogenic lymphocytes and CIN progression, CIN patients with regression of cervical cytology (cases) were matched with control patients who did not exhibit cytologic regression over the same time period (measured from initial detection of abnormal cytology). In this study, cytological regression was defined as normal cytology at two or more consecutive evaluations conducted at 3–4 months intervals. For the comparison of CD4+CD25+Foxp3 Tregs and PD1+CD4+ cells, 12 patients were enrolled in the regression group, and the median follow-up duration was 16.5 (8–33) months. Twelve pairs of follow-up time-matched patients with persistent cytological abnormalities were enrolled in the non-regression group, and the median follow-up time was 19 (9–34) months. Patients were interviewed about their smoking history and their last menstrual period.

Collection and Processing of Cervical Lymphocytes

Cervical cells were collected using a Digene cytobrush as described previously.[5] The cytobrush was inserted into the cervical os and rotated several times. The cytobrush was immediately placed in a 15-mL tube containing R10 media (RPMI-1640 medium, supplemented with 10% fetal calf serum, 100 mg/mL streptomycin, and 2.5 μg/mL amphotericin B) and an anticoagulant (0.1 IU/mL of heparin and 8 mM EDTA). After incubating the sample with 5 mM DL-dithiothreitol at 37 °C for 15 min with shaking, the cytobrush was removed. The tube was then centrifuged at 330 *g* for 4 min. The resulting pellet was resuspended in 10 mL of 40% Percoll. This mixture was layered onto 70% Percoll and centrifuged at 480 *g* for 18 min. The mononuclear cells at the Percoll interface were removed and washed with PBS. Cell viability was greater than 95%, as confirmed by trypan blue exclusion, and fresh samples were immediately used for further analyses.

Immunolabeling and Flow Cytometry

Cervical immune cell preparations were immunolabeled with fluorochrome-conjugated mouse monoclonal antibodies specific for the following human leukocyte surface antigens: a programmed death-1 marker (FITC–anti-PD-1), a phycoerythrin cyanine 5.5 (PC5.5)-conjugated helper T-cell marker (PC5.5–anti-CD4), and an allophycocyanin (APC)-conjugated IL-2 receptor marker (APC–anti-CD25). After exposure to primary surface-labeling antibodies, cells were washed twice with FACS buffer (10% fetal calf serum, 1 mM EDTA, 10 mM NaN3), permeabilized with Foxp3 Fixation/Permeabilization working solution (eBioscience, San Diego, CA, USA), and immunolabeled with the anti-intracellular antigen antibody, phycoerythrin (PE)-conjugated anti-Foxp3 marker (PE–anti-Foxp3). Cells were then washed twice with Flow Cytometry Staining Buffer (eBioscience) and resuspended in Flow Cytometry Staining Buffer. Additional aliquots of the cell preparations were labeled in parallel with appropriate isotype control antibodies. Antibodies were purchased from eBioscience and BD (Franklin Lakes, NJ, USA). Data were acquired using four-color flow cytometry on FACSCalibar (Becton-Dickinson, Texarkana, TX, USA). A minimum of 5000 CD4+ T cells was analyzed per sample. The position of CD4+ T cells was determined by CD4 vs SSC gating. We used KALUZA® Flow Analysis Software (Becman Coulter, Brea, CA, USA) for data analysis.

HPV Genotyping

DNA was extracted from cervical smear samples using the DNeasy Blood Mini Kit (Qiagen, Crawley, UK). HPV genotyping was performed using the PGMY-CHUV assay method.[22] Briefly, standard PCR was conducted using the PGMY09/11 L1 consensus primer set and human leukocyte antigen-DQ (HLA-DQ) primer sets. Reverse blotting hybridization was performed. Heat-denatured PCR amplicons were hybridized to specific probes for 32 HPV genotypes

and HLA-DQ reference samples. The virological background (HPV genotyping) of 24 patients in our study is shown in Table I. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs according to an International Agency for Research on Cancer (IARC) multicenter study.[23]

Statistical Analysis

Statistical analyses, including calculation of medians and interquartile ranges (IQRs), were performed using the commercial statistical software package JMP® (SAS, Cary, NC, USA). Wilcoxon rank sum tests or Fisher's exact tests were applied for matched pair comparisons. P-values ≤ 0.05 were considered significant.

Results

Isolation of Cervical Tolerogenic T-cell Subsets in CIN Lesions

To assess cervical tolerogenic T cells, cervical samples were collected from CIN lesions positive for any HPV genotype and fractionated over a discontinuous Percoll density gradient to remove cervical epithelial cells. Cervical lymphocytes were then isolated from the interphase between Percoll and culture medium.[5] Cervical CD4+ T cells were identified among the isolated lymphocytes using CD4 vs SSC gating. The percentages of CD4+ cervical T cells that were CD25+Foxp3+ Tregs or that were PD-1+ were determined by flow cytometry. Two representative cases are displayed in Fig. 1(a,b), respectively. The proportion of cervical CD4+ T cells that were CD25+Foxp3+ was 14.2% whereas the proportion of CD4+ T cells that displayed PD-1 was 33.6% (bold lines). Among all CIN patients, a median of 11.7% (IQR: 7.3–14.6, $n = 24$) of CD4+ cervical T cells were CD25+Foxp3+ Tregs, while a median of 30.7% (20.2–38.5, $n = 24$) of CD4+ cells expressed PD-1. The proportions of tolerogenic T-cell subsets found in cervical preparations were markedly higher than those reported in circulating peripheral blood where approximately 5% of PBMCs are CD25+Foxp3+ Tregs[24] and 5% of peripheral CD4+ T cells are PD-1+.[25] These data indicate that the cervical mucosal T cells separation technique used for these investigations isolated a population of T cells with characteristics that suggest little to no contamination by peripheral blood. Further, should small amounts of contamination occur during isolation the effect on overall results would be predicted to be minimal.

Table I Patients infected with multiple HPV types were included.

HPV type	Total numbers (%)
16	5 (16.6)
18	2 (6.6)
31	1 (3.3)
45	1 (3.3)
51	1 (3.3)
52	3 (10)
53	3 (10)
55	3 (10)
56	4 (13.3)
58	5 (16.6)
70	2 (6.6)
Total	30 (100)

Of 24 patients, 4 (16.6%) were infected with multiple types. HPVs 16, 18, 31, 33, 35, 39, 45, 51, 52, 53, 56, 58, 59, 68, 73, and 82 were defined as high-risk HPVs.

Correlation of Cervical Tregs and PD-1+ CD4+ cells in CIN Lesions with Menstrual Phase, HPV Types, Smoking History, and CIN Course

Many factors, including HPV genotypes, smoking, and other microbial infections, have been reported to associate with spontaneous regression or progression of CIN.[26] In this study, we obtained cervical Tregs from histologically diagnosed CIN patients and sought correlations between cervical Tregs and potential clinical factors, which may associate with the natural course of CIN. Patients with known, symptomatic or macroscopically visible vaginal inflammation, or sexually transmitted infections other than HPV were excluded from our study. All patients were diagnosed with CIN1-2 at the time of enrollment and followed with colposcopy and cervical cytology smears every 4 months.

To account for possible confounding factors, samples from our 24 CIN patients were reanalyzed after segregation by each of the following characteristics: menstrual phase (proliferative vs secretory), HPV genotype (high risk vs low risk), and smoking history (smoking vs non-smoking). The prevalence of CD25+Foxp3+ Tregs and of PD-1+ T cells among cervical CD4+ cells was compared between each of the