を参照されたい.

Ⅲ. 肝炎ウイルスの遺伝子型（ジェノタイプ）の決定と塩基配列の解析

肝炎ウイルスが検出されたすべての例(キャリアおよび急性感染/新規感染群）について，ウイルスのジェノタイプを決定する.

新規感染例の血清検体，およびこれと同一のジェノタイプを示す肝炎ウイルスキャリアの血清検体の両者を対象として肝炎ウイルス遺伝子の塩基配列を決定し，相同性の有無を相互に対比する.

解説

HBV感染の調査時には，HBV DNAのX～PreX～Cの領域，およびPreS2～S領域の塩基配列の両者を決定し，相互に対比することにより，ほぼその目的を達することができる[4),5)].

HCV感染の調査時には，HCV RNAのE1領域，NS5A領域，NS5B領域の塩基配列を決定し，それぞれの領域を同時に対比することにより，ほぼその目的を達することができる[6)].

HBV，HCVともに，塩基配列の決定にはダイレクトシークエンス（標的とする遺伝子領域のPCR産物を対象とした塩基配列の解析）が一般に用いられるが，必要に応じて（とくに肝炎ウイルスキャリア側の血清検体については）標的とするウイルス遺伝子領域のクローニングを行い，複数のクローンの塩基配列を決定し，新規感染例のそれと対比し，感染の因果関係を検証する.

Ⅳ. 肝炎ウイルスの新規感染者と肝炎ウイルスキャリアとの時間的・空間的接触に重点をおいたカルテ調査

肝炎ウイルスのジェノタイプ，ウイルス遺伝子の塩基配列の解析結果をもとに重点的にカルテ調査を実施すべき対象者を選別する.

感染源となった可能性あるいは蓋然性が高いと推定された肝炎ウイルスキャリアとの時間的・空間的接触に重点をおいて両者の直接・間接にわたる相互接触の可能性に関する調査を行う.

なお，上記以外の肝炎ウイルスキャリアと未感染者のカルテについても「調査委員会」が所期の目的を達成するまで（感染の終息が確認されるまで）の間は調査の対象となる.

解説

カルテ調査の対象期間は，新規感染者の「感染成立推定時」を起点として6カ月遡った時点までを目安とする．

新規感染者ごとに，この期間内における透析日時，使用したベッド，そのつど担当した医療従事者名等を時系列に従って記入した一覧表を作成する．

ウイルス遺伝子の塩基配列が近似しているか，または一致した肝炎ウイルスキャリアについても同様に一覧表を作成し，両者の直接的な接触機会，および透析用器具機材，透析に付随する手術，注射等の医療行為，医療従事者等を介した間接的な接触機会の有無等についての調査，解析を行う．

この調査を通じて，新規感染者が，当該肝炎ウイルスキャリアと直接・間接に接触する機会があったと考えられる事項，およびその可能性が否定できない事項はすべて序列をつけて列記し，後日の感染拡大の阻止，再発防止を目的とした改善指導（介入）に備える．

なお，新規感染者については，調査対象期間内における当該施設の他科への受診の有無，当該施設以外の医療機関への受診の有無についても可能なかぎり詳しく調査し，記述する．

Ⅴ．当該施設への立入調査と必要に応じた改善指導

透析医療現場の構造，設備，透析医療従事者の操作手順，当該施設の管理運営状況，などに焦点を絞り込んだ立入調査を実施し，必要に応じた改善指導を行う．また一定期間後に改善状況の確認を行う．

解説

透析医療現場の構造，設備等については，①透析ベッド相互の間隔，②患者群ごとの透析ベッド固定の有無，③透析室ごとの手洗場の設置状況，④各透析ベッドから感染性廃棄物捨場までの距離，⑤再利用器具（コッヘル，駆血帯など）の整備状況等，⑥消耗品類の整備状況，などに重点をおいた調査を行う．

透析医療従事者の操作手順等については，①操作開始前の手洗い，②手袋着脱のタイミング，③観血的処置を終えた後の感染性廃棄物の捨場に至るまでの動線，④観血的処置を終えた後の再利用器具（コッヘル，駆血帯など）の取り扱い，などに重点をおいた調査を行う．

当該施設の管理，運営については，感染防止マニュアルの作成，および活用状況，透析医療従事者に対する定期的な研修の実施状況，そ

のつど使用した透析ベッドの記録，そのつど担当した医療従事者名を含めた記録の保存状況等に重点をおいた調査を行う．

参考のために文献[6]に挙げた調査報告書に記載した透析医療現場の構造設備等，および透析医療の操作手順等に関連した立入調査時の指摘事項，指摘の意図および指導内容，指導に対する病院側対応を，原文のまま掲載する（表Ⅱ－1－1，2）．これらの改善指導は，当該施設における欠陥を指摘したというよりも，感染の危険性をより低くするためにわずかでも可能性があると考えられる感染源，感染経路を（日常業務に差し支えない範囲で），すべて遮断するという考え方に立ってなされたものである．

表Ⅱ－1－1　透析医療現場の構造，設備等

指摘事項（問題事例）	指摘の意図および指導内容	病院側対応
●プッシュ式消毒液が設けられていたが，これを用いることで完全に消毒したつもりになり，手洗いが不十分になるおそれがある．	●殺菌消毒剤と流水による手洗いの併用により，十分なウイルスの排除をはかる． →流水による手洗い場も整備・活用すること．	●水道蛇口を2カ所増設した．
●手洗い場の水道カランが，手で回して開ける方式であった．	●カランを経由する汚染のおそれがある． →水道のカランを足または腕で開ける構造とすること．	●カランをレバー式に変更した．
●廃棄箱の蓋が，手で開ける構造となっていた．	●蓋を経由する感染のおそれがある． →廃棄箱の蓋は，足で開ける構造とするかまたは蓋なしにすること．	●廃棄箱に蓋は設けない． ●使用済の透析回路等を「火バサミ」で処理する．
●透析ベッドから針捨場までの動線距離が長い．	●動線距離に比例して感染の媒介要因が増すおそれがある． →従事者は注意が必要．	●専用のワゴン車を2台配置した．
●透析ベッド間の間隔が狭い．	●隣の透析ベッドに無意識に接触するなど，感染を媒介するおそれがある．	●物理的に可能な範囲で間隔を広げた．

〔広島県C型肝炎感染調査報告書，2001〕[6]

表Ⅱ－1－2　透析医療の操作手順等

指摘事項（問題事例）	指摘の意図および指導内容	病院側対応
●針刺し後，開始作業を終えるときに，コッヘルを直接台の上に置いていた．	●台を経由する感染のおそれがある． →直接台にコッヘル等を置かないこと．	●穿刺終了後の針およびコッヘル等を，新たに設置したワゴン中の専用箱に直接収納する．
●手袋の着脱手技について，適当でない事例がみられた． ①開始前の透析機器のセット時に，手袋をはめずに透析機器を操作している例があった． ②穿刺･抜針を行う看護師が，その手袋をはめたまま透析機器を操作している例があった． ③針を廃棄する際に，素手で針に触れている例があった． ④ディスポーザブル器具・器材を運搬箱で廃棄物の捨場に持って行く際，手袋をはずしている医療従事者がいた．	●手袋の着脱のタイミングに留意が必要である． ①②観血的処置を終えた手で透析器具に触れ，その後，別の者が防御のない状態でその機器に触れた場合に，透析機器を介する感染が起きるおそれがある． ③④血液の付着した針や器具に触れるときは，手袋をつけたままでいることが感染の機会を減らすことになる． →手袋の着脱のタイミングを全医療従事者に対して徹底すること．	●原則として患者ごとに手袋の交換をする．
●経験豊かな看護師等は観血的処置等を手際よくやっており，感染防止上問題ないが，医療従事者全体では技術にバラツキがある可能性がある．	●感染防御への理解度や，観血的処置の技術レベルが低い医療従事者では，感染防止措置が不十分なおそれがある． →経験豊富な者の技術レベルに合わせて操作手順を標準化すること． →人工透析施設における感染防止マニュアルを徹底するため，定期的な研修を継続すること．	●スタッフ間での操作技術の標準化のため，内部の教育を続ける． ●人工透析施設における感染防止マニュアルを作成し，活用する．
●再利用機器をハイターに浸して消毒しているが，十分な流水洗浄がなされているか不明．	●流水洗浄が不十分なままで浸すと，表面が膜状になり，内部まで殺菌できない． →殺菌を行うに先立って，流水洗浄を十分に行うこと．	●流水洗浄を十分行う．
●透析患者が利用するベッドが，人ごとに固定化されていない．	●一つのベッドを利用する患者が増えるほど，感染範囲も広がるおそれも大きくなる． →透析患者ごとに利用ベッドを固定化すること．	●徐々に実施していく．

〔広島県C型肝炎感染調査報告書，2001〕[6]

Ⅵ. 改善指導（介入）後の効果の検証

「調査委員会」の指摘に従った改善が完了したことを確認した時点から6カ月ないし12カ月後を目安にウイルス・血清学的検査を行い，改善指導（介入）の効果を検証する．

解説

指導に基づいた改善が終了したことを確認した時点において行ったウイルス・血清学的検査（ベースライン・スタディ）を行う．この検査で肝炎ウイルスに感染していないことが確認されたすべての医療従事者，透析患者を対象として6カ月ないし12カ月を目安に検査を行い，新たな感染が起こっていないことを確認する．なお，効果の検証を終えた時点で，当該施設に対して「調査委員会」の指摘に基づき改善された感染防御水準を今後も維持，向上させ続けることの重要性と，これ以降も少なくとも1年に1回の頻度でウイルス・血清学的検査を継続する必要があるとの勧告を行うことが望ましい．

Ⅶ. 調査報告書の作成と事後指導

調査によって得られた具体的データと，これに基づいた改善指導の内容等を総括した調査報告書を作成し，当該地域の行政を通じて他の透析医療機関に情報を開示・提供し，院内感染防止に関する細心の注意の喚起と防御措置の必要性の啓発に役立てる．
なお，報告書の作成に当たっては，透析医療機関の名称，および医療従事者，透析患者の両者について個人が特定されうる記述は避けるなど，プライバシーの保護には最大限の注意を払う必要があることは言うまでもないことである．

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27. 肝炎ウイルスによる肝外病変

●a. 概念

肝炎ウイルスは，肝以外の臓器や組織にも障害を引き起こすことが知られている．これらを総称して肝外病変と呼ぶ．特にC型肝炎ウイルス(HCV)は，さまざまな肝外病変を引き起こすことが知られている(表E.44)[1,2]．ここでは，今後さらにその機序を明らかにする必要があると考えられている病態を中心に供述する．

●b. A型肝炎ウイルス（HAV）と肝外病変

○1) 急性腎不全

重症肝炎例に合併することが多いが，通常の急性肝炎例にも見られ，A型急性肝炎の1%前後に出現するといわれる．発生機序は明らかではないが，A型肝炎では血中免疫複合体が高頻度に検出され，エンドトキシンも他のウイルス肝炎に比べ高頻度に検出されることから，エンドトキシン，免疫複合体，ウイルスによる直接障害，循環不全，肝障害による代謝産物の関与など種々の要因が考えられている．

○2) 血液疾患

合併する血液疾患として，赤芽球癆，再生不良性貧血，溶血性貧血，特発性血小板減少性紫斑病などが報告されている．これらの血液疾患は重篤な合併症であり，その発生機序はまだ明らかにさ

●表E.44 肝炎ウイルスの肝外病変

HAV	HBV	HCV
急性腎不全	糸球体腎炎	クリオグロブリン血症
血液疾患	多発性動脈炎	膜性増殖性糸球体腎炎
赤芽球癆	皮膚疾患	晩発性皮膚ポルフィリン症
再生不良性貧血	Gianotti 病	Sjögren 症候群
血小板減少症	関節リウマチ	悪性リンパ腫
心筋障害	多発性筋炎	筋炎
筋炎	血液疾患	心筋障害
血管炎	赤芽球癆	口腔癌
髄膜脳炎	血小板減少症	扁平苔癬
髄膜炎	再生不良性貧血	糖尿病
Guillain–Barré 症候群		間質性肺炎
		Mooren 角膜潰瘍
		関節リウマチ

れていないが，造血細胞に対するウイルスの直接障害を示唆する報告もある．

○3) その他

心筋障害，髄膜炎，髄膜脳炎，Guillain-Barré症候群，膵炎，自己免疫性肝炎の誘発，耐糖能異常などがある．

●c. B型肝炎ウイルス（HBV）と肝外病変

○1) 腎疾患

HBVキャリアやB型慢性肝疾患では，数%～20%の頻度で蛋白尿を認め，組織学的には膜性腎症など糸球体病変の所見が得られる頻度が高い．肥厚した糸球体基底膜にHBe抗原の沈着を認めることが多く，HBe抗原と抗体の免疫複合体が糸球体に沈着することが原因であろうと考えられている．

○2) 多発性動脈炎

病因は不明であるが，10～54%にHBV感染の合併が認められる．治療法として，ステロイドを用いる方法と，インターフェロン（IFN）や抗ウイルス薬を用いる方法などがある．

○3) 皮膚疾患：Gianotti病

Gianotti病では，肝機能障害とHBs抗原が証明される．

○4) その他

関節リウマチ，Schönlein-Henoch紫斑病，多発性筋炎，血小板減少症，再生不良性貧血などがある．

●d. C型肝炎ウイルス（HCV）と肝外病変

○1) クリオグロブリン血症

混合型クリオグロブリン血症では血管壁に免疫複合体が沈着し，局所で補体系が活性化され血管炎，腎障害，紫斑，関節痛，浮腫などが発症すると考えられている．C型慢性肝炎患者におけるクリオグロブリン陽性率は高率であると報告されているが，クリオグロブリン血症とHCV genotypeには関連性を認めていない．また，クリオグロブリンそのものからHCV抗体やHCV-RNAが検出されることもわかっている．Misianiらは，IFN治療終了後，HCVの排除に伴い，血管炎の改善と血中クリオグロブリンレベルの低下を認めたと報告している．

○2) 膜性増殖性糸球体腎炎（MPGN）

HCV感染症にはMPGNの発症率が高いことが報告されている．わが国ではOhtaらが，C型慢性肝炎患者の腎病変の合併について臨床病理学的に検討を行い，腎生検953例中MPGNは12例に認められ，そのうちHCV抗体陽性は4例（33%）であり一般供血者に比べ有意に高率であったと報告している．現在IFN療法は，予後が悪いHCV-MPGN症例に対して最も効果の期待できる治療法である．

○3) 晩発性皮膚ポルフィリン症（PCT）

HCVとPCTの関連を示唆する報告が相次いでなされ，スコットランドでは，本症の91%にHCV抗体が陽性であったと報告されている．しかし，その病因論的意義については不明である．なお，HCV抗体陽性のPCTにIFNを投与することで，HCV-RNAが消失するだけでなく皮膚症状も軽快したことを示す報告がある．今後HCVによるPCTの発症機序を解明する必要がある．

○4) Sjögren症候群

Koikeらは，HCVトランスジェニックマウスにおける唾液腺炎の発現を報告した（発現率84.1%）[31]．彼らは，HCVエンベロープ蛋白発現トランスジェニックマウスでは，肝の組織変化は見られないが，涙腺，唾液腺にsialadenitisの出現を確認し，HCVとSjögren症候群類似唾液腺炎との関連を明らかにするとともに，特にHCVのエンベロープ蛋白が唾液腺炎の発症に深く関与していることを示唆した．

○5) 悪性リンパ腫

non-Hodgkin B-cell lymphoma（NHL）ではHCV感染が高いこと，そしてC型慢性肝疾患患者には腫大した腹部リンパ節がしばしば観察され，リンパ節からはHCV-RNA(+)鎖が検出されることがわかっている．Ferriらは，B-cell NHL患者50例のうちHCV感染率は34%に達し，この率はhealthy controlsの1.3%に比較しきわめて高率であったと報告している．さらに彼らは，C型慢性肝炎患者500例における悪性リンパ腫合併の発生頻度は，2.8%で（14例，diffuse B-cell NHL），14例全員に

末梢血リンパ球からHCV-RNAが検出されたと報告している．HCVはリンパ球に感染し増殖するが，HCVには逆転写酵素もなく宿主細胞のゲノムにも組み込まれず，癌遺伝子の存在も現在のところ証明されていない．

○6) 心筋障害

心筋疾患とHCVとの関連も注目されている．拡張型心筋症や肥大型心筋症において，高頻度にHCV抗体が検出されると報告されている．Matsumoriらは，拡張型心筋症の血中HCV抗体陽性率は16.7%（6/36例），肥大型心筋症では15.7%（8/51例）であり，ともに虚血性心疾患のHCV感染率（2.5%）よりも有意に高率であったと述べている．さらに彼らは，心筋生検組織や剖検心から(+)鎖とともに(−)鎖のHCV-RNAが検出されることを報告し，HCVが心筋内で増殖している可能性を示した[4]．HCV感染者は，非感染者に比して不整脈，高血圧症などをはじめとする種々の循環疾患の罹患率が高いことを示す報告もある．また，HCVと筋障害の直接的な関連は証明されていないが，筋炎とHCVを示唆する報告がある．

○7) 扁平苔癬

扁平苔癬患者には高率にHCV感染が見られる．特に，日本，イタリア，スペインでは高率で，北部九州では64.4%である．われわれの検討した扁平苔癬患者には，約8割に肝疾患を認め，このことは大規模な疫学調査からも同じ結果が得られている[5]．今までにOLPの発症にかかわるウイルス側の因子としてHCV genotypeやウイルス量との関連，OLPの免疫組織学的検討，IFN療法との関連，HCV感染を伴った難治性OLPに対する治療，組織中におけるHCVの存在と増殖などが検討されてきた[6]．いずれの結果からも，HCVはこれらの疾患の病因として重要な役割を担っていると考えられるが，さらに詳細な検討が必要である．

○8) その他

Mooren角膜潰瘍，糖尿病，間質性肺炎，間接リウマチ，皮膚病変などがある．

〔長尾由実子・佐田通夫〕

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Early Dynamics of Hepatitis C Virus in the Circulation of Chimpanzees with Experimental Infection

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Key Words
Chimpanzees · Chronic hepatitis · Hepatitis C virus · Nucleic acid amplification testing · Transfusion

Abstract
Two chimpanzees were inoculated with hepatitis C virus (HCV) and followed on a daily basis for 12 days. HCV RNA became detectable in their sera on day 5 by polymerase chain reaction with the detection limit of 10^2 copies/ml. Based on an exponential growth observed until 8 or 9 days after inoculation in their sera, the doubling time of HCV in the circulation was estimated at 6.3–8.6 h and log time (time required to grow 10-fold) at 31.3–42.9 h. The exact doubling time of HCV determined in them would help plan an efficient strategy for screening out blood donors in the window period of infection between the exposure and the development of antibody to HCV in serum.

There still remains a residual risk of contracting hepatitis C virus (HCV) infection after transfusion with blood units without the antibody against it (anti-HCV) detectable by the second- and third-generation immunoassays [1]. Anti-HCV is not raised in the circulation of individuals during the 'window period' after the exposure to HCV that is estimated at an average of 55 days in chimpanzees with experimental infection [2] and 41 days in human beings [3]. In order to identify early HCV infection, nucleic acid amplification testing (NAT) has been introduced to transfusion services [3, 4]. NAT can detect by far the most blood units in the window period of HCV infection, but cannot identify them all on a theoretical basis [5]. Even when 200 μl of serum from a donor is tested by the individual NAT, approximately 10^2 copies/ml of HCV RNA are required to produce a positive result. The sensitivity is reduced further in a mini-pool NAT performed on 50 donors in the current practice, in which a single donor is represented by merely 4 μl of serum. In actuality, HCV infection can occur in the recipient of platelet concentrates from a blood donor testing negative by NAT [6].

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The doubling time and log time (time required for growing 10-fold) of HCV in the circulation are prerequisite to planning a strategy for efficiently detecting HCV infection in blood donors as well as for understanding the limit of current screening methods based on polymerase chain reaction (PCR). They have not been determined accurately, however, due to the lack of in vitro systems for HCV culture. The documented doubling time of HCV varies widely from 2.4 h (0.1 day) [3] to 14.9 h [7] or 17.3 h (0.72 day) [5].

The dynamics of HCV growth in an early phase of infection were analyzed in the circulation of 2 chimpanzees for determining how soon HCV RNA increases to 10^2 copies/ml that can be detected by the individual NAT. Further, the doubling time and log time of HCV RNA were estimated based on the exponential growth in their sera during an early phase of infection.

Two chimpanzees entered the experimental transmission study – chimp No. 224 (C224: male, 14 years old, weight 59.1 kg) and chimp No. 267 (C267: female, 7 years old, weight 49.0 kg). They both were kept in individual cages and received humane care in accordance with all relevant requirements for the use of primates in an approved facility. Neither of them had serological or molecular virological evidence for past or present HCV infection before the inoculation. They received inocula while they were under anesthesia with ketamine hydrochloride.

Fresh-frozen plasma was obtained from a donor who was in the window period of infection with HCV genotype 1b. It was separated within 6 h after blood collection, and contained HCV RNA at a titer of 8.4×10^6 copies/ml determined by Taq Man PCR (Applied Biosystems Japan, Tokyo, Japan). The plasma was kept frozen at –80° in 1-ml aliquots; the infectious activity of the plasma in chimpanzees decreased >100-fold during these procedures [8]. An aliquot (1 ml) of this plasma was thawed in a 37° bath and injected intravenously to C224. The other chimpanzee (C267) was inoculated with a passage of another fresh-frozen plasma through a chimpanzee that had been inoculated with 1 ml of it and developed viremia [8]; the plasma was donated by an individual in the window period of co-infection with HCV genotypes 1b and 2a. Serum obtained from the chimpanzee 7 weeks after inoculation was aliquoted in 1-ml volumes and snap-frozen in liquid nitrogen. An aliquot was serially diluted 10-fold with self serum of C267, and 1 ml of a $1:10^3$ dilution containing approximately 200 copies of HCV RNA was injected intravenously to C267; it corresponded to 10 times the minimal infectious dose of HCV in chimpanzees [8]. After the inoculation, serum samples were obtained from them daily during the same hour in the morning (9–10 a.m.) for the first 12 days. They had been kept frozen at –80°, and were tested for HCV RNA simultaneously in the same assay by Taq Man PCR.

The dynamics of HCV RNA in sera from the 2 chimpanzees during an early phase of infection are illustrated in figure 1. HCV RNA was first detected in serum taken 5 days after the inoculation from them both by Taq Man PCR. Then, HCV RNA titers increased exponentially on the log scale for 8 days after inoculation in C224, and until 9 days in C267. Thereafter, HCV RNA in them deviated from the straight line of exponential growth. Based on the linearity of an initial exponential growth and the coefficient of determination, the doubling time of HCV replication was calculated to be 6.3 and 8.6 h, and the time required to grow 10-fold (log time) to be 31.3 and 42.9 h in C224 and C267, respectively; they were in a remarkably good agreement.

The growth of HCV was closely followed in the circulation of 2 chimpanzees, 1 of whom (C267) had been inoculated with passaged and calibrated HCV from a chimp in the preacute phase of infection [8]. The growth curves of HCV in the 2 chimpanzees were strikingly similar. HCV RNA was not detected for 5 days, then increased exponentially until the 8th or 9th day, and decreased thereafter. The failure in detecting HCV RNA during the initial 4 days would be attributed, in part, to a limited sensitivity of the Taq Man PCR method (10^2 copies/ml). When the linear growth was extrapolated beyond the detection of HCV RNA in serum, however, it converged to day 3 in 1 chimp and a little later than day 2 in the other (fig. 1). Hence, it would be reasonably delineated that HCV would have started circulating in both chimpanzees as early as 2–3 days after infection, a few days before HCV RNA became detectable by PCR 5 days after the inoculation.

The time from HCV transmission to the first detection of viral RNA in the circulation is called the 'eclipse' phase [3], which may vary by the size of inoculated dose and the sensitivity in detecting HCV RNA. The eclipse phase of 5 days observed in the chimpanzees (C267) inoculated with 10 times the minimal infectious dose of HCV [8] was longer than that of 3 days reported for an experimentally transmitted chimpanzee inoculated with 0.5 ml of human serum containing $10^{6.5}$ chimp infectious doses per ml [9]. Since high-dose transmission by transfusions has been excluded by anti-HCV screening [1] and low-dose infection through blood units in the NAT window period is at issue nowadays [3, 4], a longer eclipse phase would be

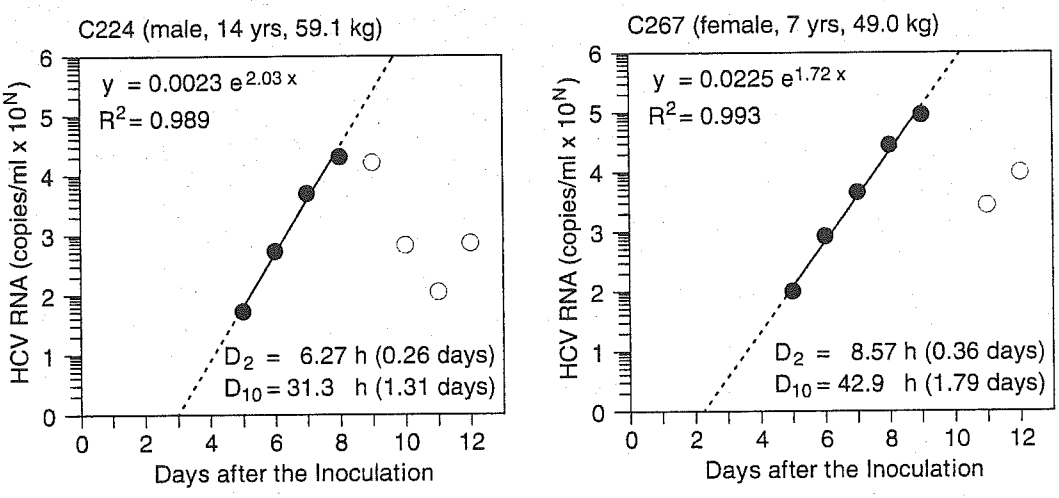

Fig. 1. Exponential growth of HCV during an early phase of HCV infection in 2 chimpanzees experimentally transmitted with pedigreed inocula. HCV RNA was determined by Taq Man PCR. Solid circles and the solid line represent the linear exponential growth of HCV. Dotted lines show an imaginary growth below the detection limit in initial days (to the left) as well as a putative exponential growth beyond the linearity (to the right). Open circles are HCV RNA levels that deviated from the linearity of exponential growth. R^2 = Coefficient of determination; D_2 = doubling time; D_{10} = the log time required for growing 10-fold.

more informative practically. An observed interval between 'true' eclipse phase (2–3 days) and PCR eclipse phase (up to 5 days), however, indicates that NAT would not be able to close the window spanning a few days after the exposure to HCV.

The duration of exponential HCV growth is called the 'ramp-up' phase [5]. Should the ramp-up phase last until all susceptible hepatocytes are infected, the slope of exponential growth would hardly be influenced by the size of HCV dose. Doubling times of HCV in the circulation calculated on the exponential growth of viral RNA in the 2 chimpanzees were 6.23 and 8.36 h, respectively. They were half as long as 14.9 h [7] and 17.3 h [5] reported in human beings. It is not clear how such a big discrepancy has arisen. Although species differences may give an account on it, a rigorous and meticulous design for experimental transmission conducted in chimpanzees would hardly be feasible for HCV infection in the transfusion setting. This issue needs to be looked into and settled, since the doubling time of HCV is crucial in working out measures for increasing the blood safety.

The results obtained in this study would help determine the size of pool in NAT for efficiently screening HCV RNA in blood donors. Due to extremely fast replication of HCV, the merit of reducing the size of pool would have its own limit. Based on the doubling time of 6.3–8.6 h in this study, the window can be narrowed by at most 1.3–1.8 days even by performing the individual NAT, in place of a mini-pool NAT on 50 donors in the current practice. This goes along with the mathematical model of Weusten et al. [5] who calculated the risk of contracting HCV infection to decrease only to one half by performing the individual NAT in comparison with a mini-pool NAT on 50 donors. Despite the doubling time of HCV, which is much shorter than hepatitis B virus (62.4 h) or human immunodeficiency virus type 1 (20.5 h) [10], there would be a limitation in narrowing the window period by performing the individual NAT. For further increasing the safety of blood transfusion, in terms of the risk for HCV infection, the other strategies would need to be considered, such as condensing HCV RNA in more amounts of serum from individual donors before performing a mini-pool NAT.

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Molecular evolutionary analyses implicate injection treatment for schistosomiasis in the initial hepatitis C epidemics in Japan

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***Background/Aims*: The mortality due to hepatocellular carcinoma (HCC) has ranged widely in various areas of Japan since 30 years ago and the incidence was particularly high in once *Schistosoma japonicum* (*Sj*)-endemic areas. Our aim was to estimate the spread time of hepatitis C virus (HCV) infection in the past with possible relevance to a higher incidence of HCC in once *Sj*-endemic than *Sj*-nonendemic areas.**

***Methods*: During 2001, 131 strains of HCV-1b were obtained from patients in three previously *Sj*-endemic areas, as well as *Sj*-nonendemic areas in Japan and a cross-sectional study was conducted on them with molecular evolutionary analyses.**

***Results*: A phylogenetic tree reconstructed on HCV-1b sequences in the NS5B region disclosed 2 independent clusters for *Sj*-positive and -negative groups with a high bootstrap value. The estimated effective number of HCV-infections indicated a transition from quiescence to rapid exponential growth in the 1920s among patients with schistosomiasis, which is 20 years earlier than that among patients without schistosomiasis.**

***Conclusions*: The estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* since 1921. A high incidence of HCC there would be attributed to a long duration of HCV infection since 1920s.**

***Keywords*: Hepatitis C virus; *Schistosoma japonicum*; Molecular evolutionary analysis; Hepatocellular carcinoma**

1. Introduction

Recently, the molecular clock has been successfully applied to long-term serial serum samples containing hepatitis C virus (HCV) from the US and Japan and estimated the spread time of HCV in the 1930s in Japan, which is 30 years earlier than that in the US in the 1960s [1]. Insofar as a long duration of HCV infection is the most important factor for the development of hepatocellular

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Abbreviations HCV, hepatitis C virus; Anti-HCV, antibody to HCV; HCC, hepatocellular carcinoma; *Sj*, *Schistosoma japonicum*.

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carcinoma (HCC), it can be predicted that the incidence of HCC will increase in the US over the next 2–3 decades. Thus, a combination of classical epidemiological approaches and molecular evolutionary analyses would be particularly useful in the study of contagious diseases, typified by HCV infection.

The way how individuals contracted HCV infection has remained unclear in Japan. Recently, a Japanese report (Ministry of Health, Labour and Welfare: Distribution of age-adjusted mortality rate from liver cancer by prefecture between 1971 and 1975, Tokyo, 2001) indicated that the mortality due to HCC has already varied widely in various areas of Japan since 30 years ago; the incidence of HCC was much higher in Saga/Fukuoka, Hiroshima and Yamanashi Prefectures, which were once endemic for schistosomiasis japonica in the long past. Hence, a high incidence of HCC in the 1970s would be related to HCV transmitted by injection treatment for *Schistosoma japonicum* (*Sj*) conducted since 1921 in these areas. In fact, shared needles and syringes for intravenous injection treatment with antimonyl potassium tartrate or sodium antimony tartrate posed a significant risk for HCV transmission in endemic areas [2]. Indeed, the prevalence of antibody to HCV (anti-HCV) is high (36.5; 95% CI = 28.1–44.9%) in patients with chronic schistosomiasis [2] and therefore, HCV infection is considered responsible for the development of HCC in patients with chronic schistosomiasis.

Since, once popular intravenous injection for schistosomiasis was a risk factor for HCV transmission, the spread time of HCV in the areas once endemic for *Sj* in Japan would deserve determination. In this study, molecular evolutionary analyses using principles of both population genetics and mathematical epidemiology [3] were applied to HCV-infected patients with and without a past history of chronic schistosomiasis in once *Sj*-endemic areas.

2. Meterials and methods

2.1. Sample collection

In Japan during 2001, 181 random serum samples positive for anti-HCV were obtained from patients with chronic liver disease in widely separated areas previously endemic for *Sj*, including Kofu in Yamanashi ($n = 75$), Katayama in Hiroshima ($n = 50$) and Chikugo in Saga/Fukuoka Prefectures ($n = 56$). Schistosomiasis was diagnosed by ultrasonographic (US) and/or computer tomographic (CT) modalities or serological examinations [4]. Two kinds of serological tests, which can detect past history of schistosomiasis, were available in this study. In brief, IgG antibodies binding to two different *schistosome antigens*, *Sj* adult worm antigen and *Sj* egg antigen, were detected using an enzyme-linked immunosorbent assay (ELISA). As it is now accepted that ELISA titer of egg antigen-specific IgG is reliable for case-detection rather than IgG for adult worm antigen [4–6], the results based on the egg antigen-specific IgG were accepted in this study. Samples of more than 0.25 of optical density at 415 nm were determined to be positive, as previously confirmed [4–6]. The serum samples were tested for anti-HCV by Lumipulse II Ortho HCV (Ortho-Clinical Diagnostics K.K., Tokyo, Japan). As patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. For a cross-sectional study, 30 serum samples were obtained from patients infected with HCV in Aichi Prefecture where *Sj* has not been endemic. The age- and sex- matced patients were also selected from the *Sj*-nonendemic areas excluding influece of these factors on HCC incidence. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by Ethic Committees of institutions. Every patient gave a written informed consent to participate in the virological research of HCV. Information of injection treatment for *Sj* was obtained by means of self-administrated questionnaires or structured interviews. None had been treated with interferon therapy for HCV infection. HCC incidence was estimated by historical information from patients themselves and/or medical records during 2001. HCC was diagnosed by liver biopsy or combination of imaging modalities such as US, enhanced CT and angiography.

2.2. Genotyping and sequencing

Nucleic acids were extracted using a SepaGean RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd., Tokyo, Japan) in accordance with the manufacturer's protocol. They were reverse-transcribed to cDNA using SuperScript II Rnase H^- Reverse Transcriptase (Invirogen Corp., Carlsbad, California, USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) by the method described previously [7].

A sequence spanning 339 nucleotides (nt) in the NS5B region was amplified by polymerase chain reaction (PCR) with primers described previously [1]. PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, California, USA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in PCR, PLATINUM Pfx DNA Polymerase (Invirogen Corp.) with a very high fidelity was used. The sequences determined were utilized to confirm HCV genotypes and construct phylogenetic trees.

2.3. Test for clustering between Sj-positive and -negative groups

The phylogenetic tree was first constructed to examine the evolutionary history for *Sj*-positive and *Sj*-negative groups by the neighbor joining method [8]. Furthermore, to test whether either *Sj*-positive or *Sj*-negative group have evolved independently or not, we conducted an interior branch test for the neighbor-joining tree [9]. Thereafter, a *t*-test was conducted for the interior branch length and its standard error, which is computed using the bootstrap procedure.

2.4. Demographic model

A reconstructed tree was built on the NS5B sequence of 339 nt by a heuristic maximum-likelihood topology search with stepwise-addition and the nearest neighbor-interchange algorithms. Tree likelihood scores were calculated using HKY85 with the molecular clock enforced by PAUP version 4.0b8.

As estimates of the demographic history, a nonparametric function $N(t)$, known also as the skyline plot, was obtained by transforming coalescent intervals of an observed genealogy into a piecewise plot that represents an effective number of infections through time [3,10]. A parametric maximum-likelihood was estimated by several models with the computer software Genie v3.5 to build a statistical framework for inferring the demographic history of a population on phylogenies reconstructed on sampled DNA sequences [10]. This model assumes a continuous epidemic process in which the viral transmission parameters remain constant through time. Model fitting was evaluated by likelihood ratio tests of the parametric maximum-likelihood estimates [11,12].

2.5. Statistical method

Data for continuous variables were demonstrated as the mean ± standard deviation. The Fishers' exact test, Chi square test with Yates' correction and one-way ANOVA followed by the Scheffe's multiple comparison test were used to evaluate differences in the mean age, sex ratio

and incidence of HCC between groups, respectively. Differences with P values less than 0.05 were considered significant.

3. Results

Of 181 anti-HCV positive samples, 113 were classified into HCV genotype 1b (HCV-1b), which is predominant in Japan. Fifty-two of 181 samples (29%) were negative for HCV RNA or incomplete for sequencing and the remaining 16 samples (9%) of genotype 2a were excluded in this study due to a minor population. Of the HCV-1b strains, 47 were recovered from patients in Yamanashi, 31 in Hiroshima and 35 in Saga/Fukuoka Prefectures. Along with 18 HCV-1b strains in Aichi Prefecture serving as controls, a cross-sectional study was conducted on them with molecular evolutionary analyses. The patients in areas previously endemic for *Sj* revealed a significantly higher prevalence of chronic schistosomiasis [24/47 (51%) in Yamanashi (Kofu area), 21/31 (68%) in Hiroshima (Katayama area) and 19/35 (54%) in Saga/Fukuoka (Chikugo area)] than that in Aichi Prefecture (0/18 [0%], $P<0.0001$). There were no significant differences in the mean age or sex ratio among patients from these four areas (Fig. 1). Although the mean age of *Sj*-positive patients was just higher than that of *Sj*-negative patients in once *Sj*-endemic areas or matched-control patients in Aichi Prefecture, there were also no significant differences between these groups (Table 1).

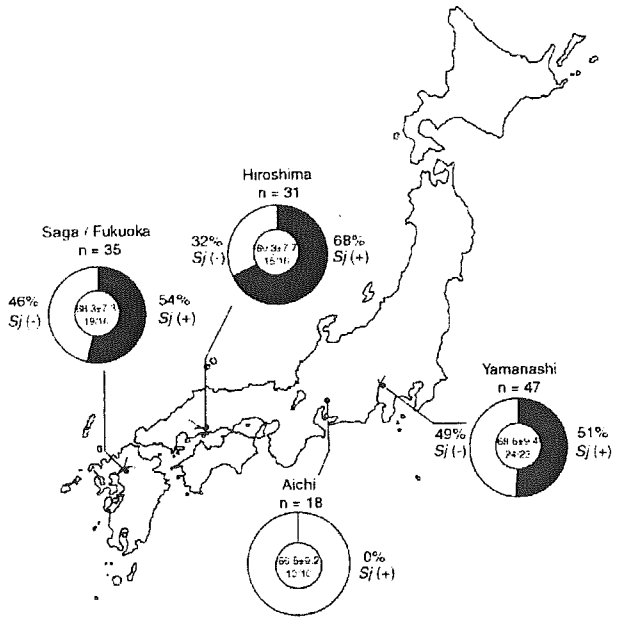

Fig. 1. Geographic distribution of *Schistosoma japonicum* (*Sj*) and characteristics of patients infected with HCV. *Sj* (+) and *Sj* (−) denote, respectively, presence and absence of infection with *Sj* diagnosed by ultrasonographic and/or computer tomographic methods or serological examinations. Pie graphs include the age (mean ± standard deviation) and sex ratio (male/female).

Table 1
Characteristics of patients with and without schistosomiasis

	Schistosoma japonicum		Controls
	Positive ($n=64$)	Negative ($n=49$)	(Aichi) ($n=18$)
Mean age			
Total	69.9 ± 7.7	67.4 ± 8.7	66.5 ± 9.2
Yamanashi	69.9 ± 7.2	67.3 ± 11.2	
Hiroshima	71.2 ± 8.7	67.6 ± 6.5	
Saga/Fukuoka	69.0 ± 7.7	67.5 ± 7.1	
Sex (male/female)			
Total	34/30	24/25	9/9
Yamanashi	13/11	11/12	
Hiroshima	10/11	5/5	
Saga/Fukuoka	11/8	8/8	
Incidence of HCC	25/55 (45%)	11/48 (23%)	3/18 (17%)

The incidence of HCC in *Sj*-positive patients was significantly higher than that in *Sj*-negative patients ($P=0.0226$) or controls ($P=0.0488$).
Abbreviations: HCC, hepatocellular carcinoma.

For cross-sectional study on the viral population size between HCV-infected patients with and without a past history of schistosomiasis, a phylogenetic tree for HCV-1b strains in the *Sj*-positive and -negative patients was constructed with use of the maximum-likelihood method enforced by the molecular clock as introduced in our previous report [1] and an independent study by Pybus et al. [3]; a substitution rate of 5.3×10^{-4} per site per year [1,3] was assumed for HCV. The phylogenetic tree disclosed 2 independent clusters for *Sj*-positive and -negative groups, with a high bootstrap value (81%) by the interior branch testing (Fig. 2), which is comparative with past epidemiological backgrounds in Japan. From distinct evolutionary histories in the two populations, the effective number of HCV-1b infections through time, $N(t)$, were assessed by the skyline plot. The parameters for several models in Genie v3.5 [3,10] were also examined. Time t was then transformed to year using the same rate, assuming the collecting time (year 2001) as the present. Fig. 3 shows the skyline plots and population growth for *Sj*-positive and -negative patients, according to a specific demographic model in Genie v3.5 with three parameters, piecewise expansion growth model, that was evaluated by the likelihood ratio testing [11,12]. Molecular evolutionary results thus obtained supported our previous study in which the divergence time of the most recent common ancestor of HCV-1b in each area in Japan was estimated before 1850 [1]. Our estimates of the effective number of HCV-infections showed a transition from constant size to rapid exponential growth in the 1920s among patients with chronic schistosomiasis in endemic areas, which is 20 years earlier than that among patients without schistosomiasis in the 1940s. Information on HCC was available for 121 of the 131 patients with HCV-1b. Although they were relatively small in number, the incidence of HCC was significantly higher in *Sj*-positive than -negative patients ($P=0.0226$) or controls ($P=0.0488$) (Table 1).

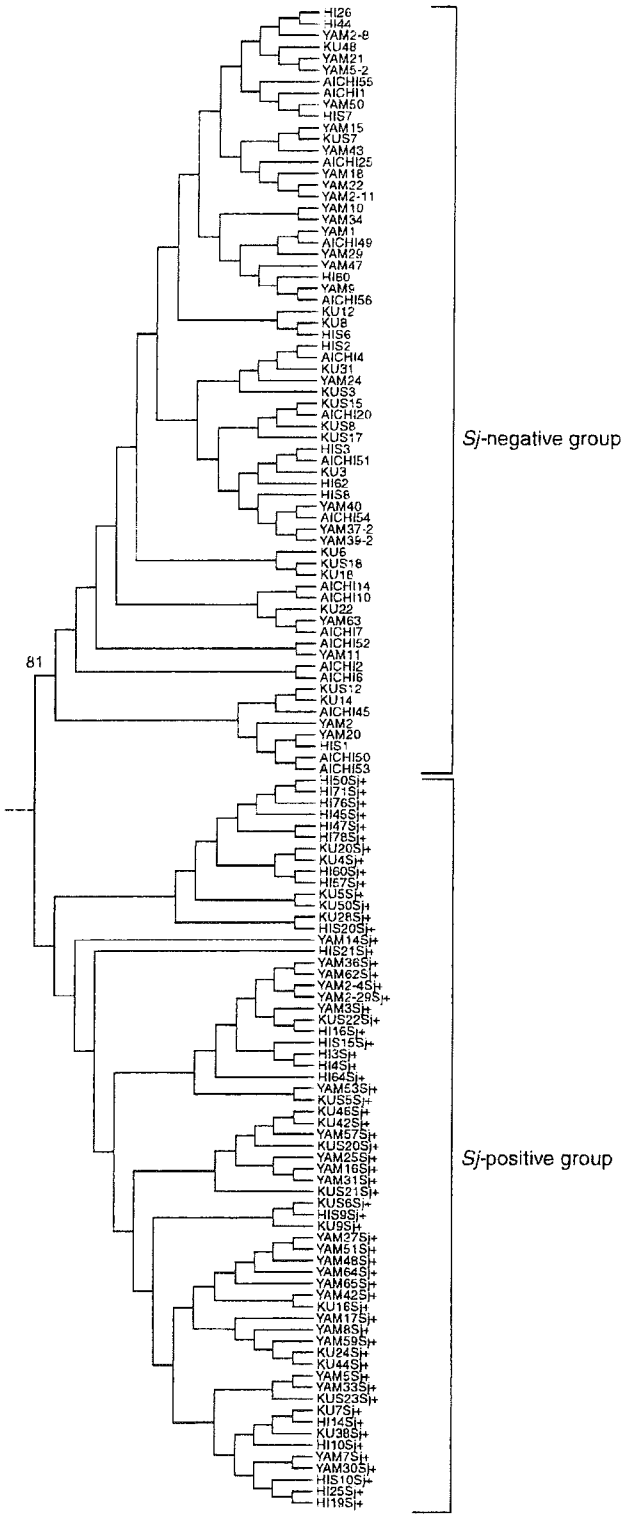

Fig. 2. A phylogenetic tree constructed on NS5B sequences of HCV-1b strains in *Schistosoma japonicum* (*Sj*)-positive ($n=64$) and -negative ($n=67$) groups. The numbers in the tree indicate bootstrap reliability by the interior branch test. *Sj*+ indicates *Sj*-positive strains. YAM; Yamanashi, HI/HIS; Hiroshima, KU/KUS; Saga/Fukuoka, Aichi; control strains.

4. Discussion

The specific demographic model based on the neutral theory [3,11,12], which has a constant size in the past and changes to exponential growth until the present, is applied to investigate the Japanese endemic of HCV. By means of the molecular evolutionary analyses, the spread time of HCV in *Sj*-positive patients was estimated 20 years earlier than that in *Sj*-negative patients from three areas in Japan where *Sj* was previously endemic (Yamanashi, Hiroshima, Saga/Fukuoka Prefectures). The spread time of HCV much earlier in *Sj*-positive than -negative patients indicates that the previous intravenous injection treatment with antimony compounds (antimonyl potassium tartarate or antimony sodium tartarate) on patients with schistosomiasis since 1921 [2] would have been a significant risk factor for HCV transmission in endemic areas through re-used needles and syringes. Indeed, it might be possible that HCV transmission from *Sj*-positive patients to *Sj*-negative patients occurs in the once *Sj*-endemic areas, but we could not find such strains in this study. One of the reasons is that residents in the village around the river, where schistosomiasis had been the most prevalent, might have been isolated from those in the other areas of the same Prefecture in the past due to the endemic disease 'schistosomiasis'. Interestingly, most Japanese strains from *Sj*-nonendemic areas in the database clustered with the *Sj*-negative group of the present study. Hence, factors other than the injection treatment for *Sj*, such as intravenous stimulants popular during and after World War II [13] and medical treatments including transfusion with blood units from paid donors in the past, would have imposed the risk for HCV transmission in most areas in Japan [14]. In addition, there would have been opportunities for HCV transmission through inadequately sterilized needles and syringes in general practices, which have contributed to a large reservoir of chronic HCV infection in Japan during the 1950s [13]. Such inadequately sterilized medical injections were still common in the less-developed world in the 20th century. WHO estimates that unsafe injections result in 2.3–4.7 million new HCV infections worldwide every year [15].

Although the spread time of HCV in *Sj*-positive group was earlier than that in *Sj*-negative group, there was no significant difference of mean age between the 2 groups. Two possibilities are considered. One is a sampling bias; as patients with *Sj* treatments were estimated to be old, relatively older patients were selected in the *Sj*-endemic areas to match age factor that might influence duration of HCV infection or HCC incidence. Second, the ages that patients had been infected with HCV were different between the 2 groups; the treatments for *Sj* in Japan were mainly conducted among relatively younger people including school children after screening of *Sj* [4,16,17], while the

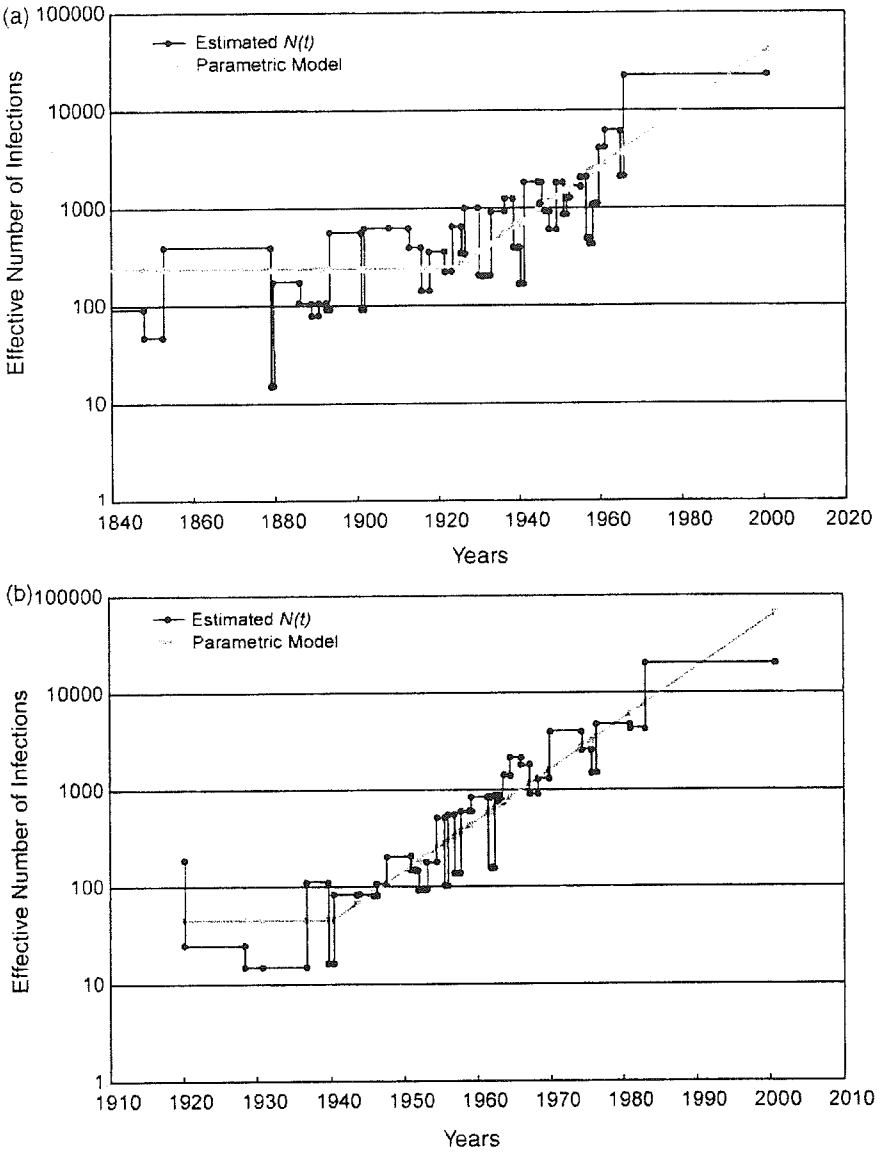

Fig. 3. The maximum-likelihood estimates of $N(t)$ on the effective number of infections with HCV genotype 1b in Japan for *Schistosoma japonicum* (*Sj*)-positive group (a) and *Sj*-negative group (b) separated in the phylogenetic tree (Fig. 2). The parametric model is indicated by the grey line and stepwise plots by the black line that represents corresponding nonparametric estimates of $N(t)$ (number as a function of time). Genetic distances are transformed into a time scale of year using estimates of the molecular clock in the NS5B region.

other risk factors such as blood transfusion were found in older people excluding at least children.

A disease possibly caused by schistosomal infection in Japan is documented in a book written some 300 years ago. In 1847, the clinical picture of this disease was precisely described by Yoshinao Fujii in the book 'Katayama-ki' that documented an endemic disease in Katayama area as Katayama's disease (equivalent to schistosomiasis). Water-borne epidemics of schistosomiasis prevailed in inhabitants around rivers (the tributaries of the Fuji river in Yamanashi, the Takaya river in Hiroshima and the Chikugo river in Saga/Fukuoka) in Japan, mediated by small shellfish (Miyairi-kai) serving as the natural host. More than 200,000 individuals were estimated to have been infected with *Sj* in Yamanashi Prefecture alone during 1965 through 1990 [16] and approximately 1,000,000 patients in the entire Japan since 1920s [17]. To cope with these endemics, more than 10 million intravenous injections with antimony compounds had been given in Japan since 1921 [17]. Thus, Japan would have started ahead of any other countries, in terms of HCV spread in association with schistosomiasis, wherein intravenous drugs were invented. Although acute schistosomal infection has disappeared in Japan since long ago, there are still elderly people with

chronic schistosomiasis in previously endemic areas, some of whom are developing HCC [2,14]. Substantial transmission among regions is supported by the lack of regional clustering of HCV sequences in this study.

A similar situation is reported in the Nile delta in Egypt where schistosomiasis once prevailed mediated by small shellfish [18] and the national campaigns for injection treatment with antimonyl potassium tartarate (tartar emetic) from the 1961 until 1986 are suspected to have given rise to the highest endemicity of HCV in the world ever, involving >20% of the national population there [19]. The prevalence of anti-HCV is extremely high (>70%) in patients with schistosomiasis there [18,20,21]. Highly prevalent HCV infection in the general Egyptian population accounts for most HCC cases in Egypt [22]. A question may arise whether schistosomiasis alone is responsible for the development of HCC. Patients co-infected with HCV and *Schistosoma mansoni* (*Sm*) may have a high incidence of viral persistence, accelerated fibrosis and development of HCC [23,24]. A recent population-based study between two large populations with district histories of *Sm* and hepatitis C infections, however, failed to indicate any interaction between *Sm* infection and the prevalence or severity of hepatitis C [25]. Moreover, no significant histological differences were found between anti-HCV-positive Egyptian patients with and without schistosoma [26]. Hence, the long duration of persistent HCV infection would be a more important factor for the development of HCC than the pathogeneticity of *Sm* itself.

Estimating the effective number of HCV infections has been very informative in looking back epidemic spreads of HCV infection in the Unites States [1] and Egypt [12,27]. In addition, it would also be useful in predicting the population size and extent of HCV infection. Studies to foresee future spreads of HCV would be required to cope with and prevent healthcare problems where de novo infections are increasing. The advantage of molecular evolutionary analyses, its ability to accurately estimate the dynamics of HCV based on a limited number of isolates in particular [3], will extend its application anywhere in the world where clinical sequelae of persistent HCV infection pose increasing burdens on the public health of nations.

In conclusion, the evolutionary analyses indicated that the estimated spread time in previously *Sj*-endemic areas in Japan coincides with injection treatment for *Sj* conducted since 1921. The high incidence of HCC in *Sj*-endemic areas is most likely attributed to long duration of HCV infection there transmitted through injection treatments.

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