表2．各細胞レベルで一度は実施するべきウイルス試験

	MCB	WCB	CAL*1
レトロウイルス及び内在性ウイルス試験			
感染性試験	＋	－	＋
透過型電子顕微鏡観察（TEM）	＋	－	＋
逆転写酵素活性*2	＋	－	＋
その他の細胞種特異ウイルス試験*3	適宜実施	－	適宜実施
非内在性ウイルス又は外来性ウイルス試験			
In vitro 感染性試験	＋	－	＋
In vivo 感染性試験	＋	－	＋
抗体産生試験	＋	－	－
その他の細胞種特異ウイルス試験*4	＋	－	－

*1：CAL（Cells at the limit），In vitro 細胞齢の上限まで培養された細胞。
*2：レトロウイルス感染性試験が陽性の時は不要。
*3：細胞株個々の起源・由来から存在が予測されるウイルスの検出に適した試験。
*4：ヒト由来細胞株，ヒト以外の霊長類由来細胞株，げっ歯類以外の動物由来細胞株の場合，各細胞株に適切な試験を適宜実施する。

3.3.1 レトロウイルス及び内在性ウイルス試験

(1) 感染性試験

レトロウイルスの感染性試験としては，指標細胞としてラット XC 細胞を用いた XC プラークアッセイ（同種指向性レトロウイルスの検出），ミンク S+L-細胞を用いた S+L-フォーカスアッセイ（異種，両種指向性レトロウイルスの検出）を実施する。試験は被験細胞との共培養を行うか，細胞の培養上清を用いて感染させ，一定期間培養後に XC 細胞ではプラークの形成，S+L-細胞ではフォーカスと呼ばれる特有の細胞凝集塊の形成を顕微鏡で観察する。

(2) 透過型電子顕微鏡観察（TEM）

細胞の超薄切片及び培養上清を検体として電子顕微鏡で観察することにより，ウイルス及びウイルス様粒子を検出することができる。レトロウイルス以外のウイルス粒子も検出できるが，感度は高くない。

(3) 逆転写酵素活性試験

レトロウイルスが RNA を DNA に変換する逆転写酵素を持っていることを利用し，培養上清の逆転写酵素活性の測定によりレトロウイルスを検出する方法である。試験では，試料に RNA とプライマーを添加し，逆転写反応による cDNA の生成を検出する。生成された cDNA をリアルタイム PCR により増幅して検出する高感度逆転写酵素活性試験（PERT）が簡便で高感度な方法として用いられている。

3.3.2 非内在性ウイルス及び外来性ウイルス試験

(1) In vitro 試験

広範囲のウイルスを検出することができる指標細胞を用いる方法で，ウイルス感染による細胞変性効果（CPE）を指標とする方法や赤血球凝集で判定する方法がある。指標細胞には，ヒトウイルスに感受性があり広範囲のウイルスが感染する霊長類由来 Vero 細胞やヒト MRC- 5 細胞などの複数の細胞を用いる。動物由来ウイルスの混入の可能性がある場合は，動物由来細胞を用いた試験を追加する。試験は，培養細胞にウイルスを含む試料を添加して 2 週間培養し，その培養上清又は細胞破砕液を新しい細胞で継代してさらに 2 週間培養後，ウイルスの感染により出現する CPE を観察する。CPE の形態はウイルスの種類により異なる（図 4）。赤血球凝集反応は CPE をおこさないウイルスを検出するために用いる。

(2) In vivo 試験

In vivo 試験は，in vitro 試験では検出できないウイルスを検出するための試験であり，発育鶏卵，成熟マウス，乳飲みマウス，モルモット等に細胞溶解液又は培養液を接種して行う。具体的な試験方法については省略するが，動物への接種では感染による死亡，発育鶏卵への接種では尿膜腔液の血液凝集反応を指標としてウイルスを検出する。

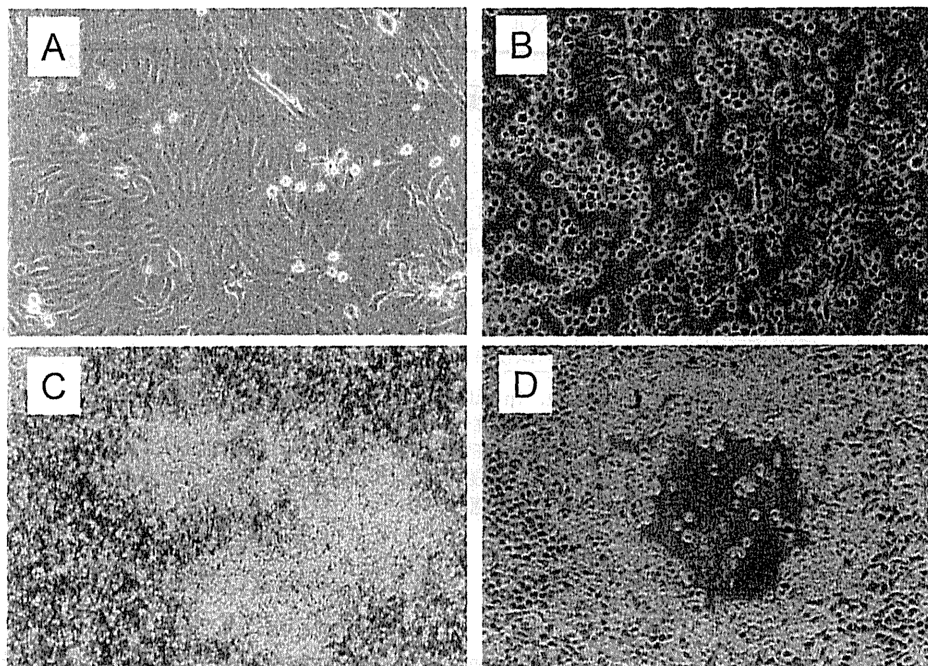

図4．ウイルス感染による細胞変性効果（CPE）の例
A：感染前の Vero 細胞。
B：Vero 細胞に HSV-1を感染後48時間の様子。CPE により細胞が丸くなり浮き上がっている。
C：ポリオウイルスに感染した Vero 細胞のプラーク。
D：ウシ水胞性口炎ウイルスに感染した Vero 細胞のプラーク。
（写真 C, D 提供：日本ケミカルリサーチ）

(3) 抗体産生試験

げっ歯類由来細胞株に存在する可能性がある種特異的ウイルスに対する試験として，マウス抗体産生試験（MAP），ラット抗体産生試験（RAP），ハムスター抗体産生試験（HAP）などを実施する。ウイルスフリーの動物に細胞溶解液又は培養液を接種し，一定期間後，被験動物の血清中に抗体産生が認められるかどうかによりウイルスの有無を判定する。

4．適切な製造段階でのウイルス否定試験の実施

細胞株由来のタンパク質性医薬品の場合，原材料は細胞培養後にハーベストされた細胞や培養上清となる。医薬品製造基材となる細胞バンクレベルで徹底したウイルス試験を行っても，培養中に外来性ウイルスの迷入により汚染が起こる可能性がある。ウイルスが迷入した場合，未加工・未精製バルクに最も高濃度で存在する可能性があるため，可能な限り未加工・未精製バルクについてウイルス試験を実施することが望ましい。未加工・未精製バルクで一般的に用いられる試験は，一種類又は数種類の細胞株を用いる in vitro でのスクリーニング試験であり，最低3ロットに対して実施する必要がある。外来性ウイルスが検出されたバルクハーベストは医薬品製造に用いるべきではないとされる。

最終製品や精製バルクでのウイルス試験の必要性は，原材料や医薬品製造基材の種類及びこれらに対するウイルス検査結果，製造工程におけるウイルスの除去・不活化の評価試験の結果，及び製造工程におけるウイルス迷入の可能性がどの程度あるかなどに依存する（表3）。げっ歯類のレトロウイルスまたはレトロウイルス様粒子のみが細胞又は未加工・未精製バルクに確認された場合（ケースB）では精製バルクにおいて当該ウイルスに対する高い特異性と感度を有する検出系を用いて試験を行う必要があるが，CHO，C127，BHK 等の細胞株の内在性レトロウイルス様粒子のように，十分に解析されており，また後述のクリアランス試験により適切なクリアランスが示さ

表3．ウイルスクリアランス工程評価と精製バルクでのウイルス試験実施要領（ICH Q5Aより抜粋）

	ケースA	ケースB
［細胞や未精製バルクでのウイルス試験結果］		
ウイルスの存在	−	−
ウイルス様粒子の存在	−	−
レトロウイルス様粒子の存在	−	+
ウイルスの分離同定の可否	適用外	+
ウイルスのヒトへの感染性	適用外	−
［必要な対応］		
ウイルスクリアランスに関する工程特性解析試験（非特異的モデルウイルスを用いたクリアランス能評価）	必要	必要
ウイルスクリアランスに関する工程評価試験（特異的モデルウイルスを用いたクリアランス能評価）	不要	必要
精製バルクでのウイルス否定試験	適用外	必要*

*: 内在性レトロウイルス様粒子が十分に解析され，適切なクリアランスが示されている場合のCHO細胞などの例では，非病原性レトロウイルス様粒子に関する試験は，通常，不要

れている場合は，精製バルクでの試験は通常不要とされている[4]。

5．製造工程へのウイルス除去・不活化工程の導入

生物薬品の製造工程に適切なウイルス除去・不活化工程を導入することにより，ウイルス安全性を高めることができる。代表的なウイルス除去・不活化工程を表4に示した。生物薬品，特にタンパク質性医薬品の場合，強力なウイルス不活化工程を導入することはタンパク質の変性，失活につながるため難しい。一方，ウイルス除去工程としてのウイルスろ過膜の性能は飛躍的に向上しており，最も小さいマウス微小ウイルス（MMV）などでも十分なクリアランス能が得られるようになっている。生物薬品のウイルス安全性を高めるためには，製造工程に機序の異なる複数のウイルス除去・不活化工程を導入することが必要である。

表4．代表的なウイルス不活化・除去工程

① 加熱処理（55～60℃，30分の処理で多くのウイルスは不活化。血液や尿由来では液状60℃，10～24時間処理を行うこともある。）
② 有機溶媒/界面活性剤（S/D）処理（脂質膜を有するウイルスに有効）
③ 膜ろ過（15～40nm）
④ 酸性処理
⑤ 放射線処理（γ線照射）
⑥ カラムクロマトグラフィー（アフィニティークロマトグラフィー，イオン交換クロマトグラフィー等）
⑦ 沈殿分画（有機溶媒分画，硫安分画等）
⑧ 抽出処理

6．ウイルスクリアランスに関する工程評価試験

ウイルスクリアランス工程評価試験は，生物薬品の各製造工程がどの程度のウイルス除去・不活化能を持つかを定量的に評価するもので，原材料へのウイルスの混入や製造工程での迷入が起こった場合でも，その後の製造工程によりウイルスを十分に除去・不活化できることを証明するのに必要である。必ずしもすべての工程について実施する必要はなく，十分に有効な除去・不活化能を持つと考えられる機序の異なる工程を2つ以上選んで評価すればよい。クリアランス試験では，評価すべき工程に一定量のウイルスを意図的に添加（スパイク）し，工程処理によりどの程度ウイルス量が減少したかを定量的に評価する。試験には性質（DNAウイルスかRNAウイルスか，エンベロープの有無，ウイルスサイズ，物理的化学的処理への耐性）の異なる複数のモデルウイルスを使用する（表5）。原料に存在する可能性のあるウイルス，例えばバイオ医薬品の製造に繁用されるげっ歯類細胞のCHO細胞には内在性レトロウイルス様粒子の存在が確認されているので，マウス白血病ウイルス（MuLV）などを「特異的モデルウイルス」として用いる。複数のモデルウイルス候補がある場合は，より耐性が強いものを選択する。高タイターのウイルスが得られるものがモデルウイルスに適しているが，高濃度でウイルス凝集が起こると除去工程が過大評価される可能

表5．ウイルスクリアランス試験に用いられるウイルスの例

ウイルス名	宿主	ゲノム	エンベロープ	サイズ(nm)	形状	耐性*
水胞性口炎ウイルス	ウシ ブタ ウマ	RNA	有	70×150	弾丸形	低
パラインフルエンザウイルス	多種	RNA	有	100～200超	多様／球形	低
マウス白血病ウイルス（MuLV）	マウス	RNA	有	80～110	球形	低
HIV-1 （HIV1,2 モデルウイルス）	ヒト	RNA	有	100～120	球形	低
シンドビスウイルス	ヒト	RNA	有	60～70	球形	低
ウシ下痢症ウイルス（BVDV） （HCVモデルウイルス）	ウシ	RNA	有	50～70	多様	低
仮性狂犬病ウイルス（PRV） （HBV，HSV モデルウイルス）	ブタ	DNA	有	120～200	球形	中
ポリオウイルス Sabin 1型	ヒト	RNA	無	25～30	正20面体	中
脳心筋炎ウイルス	マウス	RNA	無	25～30	正20面体	中
レオウイルス3型	多種	RNA	無	60～80	球形	中
SV40	サル	DNA	無	40～50	正20面体	高
イヌパルボウイルス ブタパルボウイルス （HAV，B19 モデルウイルス）	イヌ ブタ	DNA	無	18～24	正20面体	高

＊物理的・化学的処理に対する抵抗性

性や，不活化工程が過小評価される可能性もあるので注意が必要である。モデルウイルスの選択には，試験従事者の安全確保も考慮する必要がある。ウイルス汚染を引き起こすため，実際の製造工程をそのままウイルスクリアランス評価に用いることはできないが，可能な限り実製造を反映してスケールダウンした工程について評価を行う必要がある。

6.1. ウイルスクリアランス能の評価法の概略

①試料に一定量のモデルウイルスを添加（スパイク）する。

②評価したい製造工程によりモデルウイルスを添加した試料を処理する。

③工程処理前後の試料溶液に含まれるウイルス量を感染性試験やNATにより定量的に評価する。

④ウイルスクリアランス指数（ウイルス力価の減少度を対数で表したもの：LRV）を以下の式により求める。

$$LRV = Log [(V1 \times T1)/(V2 \times T2)]$$

V 1：工程処理前の試料の容量

T 1：工程処理前のウイルス濃度（力価）

V 2：工程処理後の試料の容量

T 2：工程処理後のウイルス濃度（力価）

たとえば，ウイルス力価 10^8IU/ml の試料 1 ml をカラムにアプライし，得られた 10ml の溶出液中のウイルス力価が 10^3IU/ml であった場合，LRV は 4 となる。一般に，4 以上のクリアランスが得られる工程を有効な頑健性のあるウイルス除去・不活化工程として評価することができる。製造工程全体のウイルスクリアランス能は，各工程のLRVの合計により算出するが，機序が同じ工程は合算できない。図1の製造工程に当てはめた表6の①，②のケースでは，総 LRV は①のほうが高いが，①の製造工程には十分なクリアランスが得られない工程が含まれ，特に LRV が 1 以下の工程を合算することは妥当ではない。②のケースでは LRV 4 以上の工程が 3 つ含まれ，

表6．ウイルスクリアランス工程評価の例

製造工程	LRV	
	ケース①	ケース②
クロマトグラフィーA	2.7	ND
酸処理	3.5	4.2
クロマトグラフィーB	2.1	4.0
クロマトグラフィーC	0.9	ND
ナノフィルトレーション	4.5	4.3
総 LRV	12.7	12.5

総 LRV は12.5としてよい。CHO 細胞などでは内在性のレトロウイルス様粒子が存在（$>10^8$）するが，表6がレトロウイルスのクリアランス評価結果の場合，安全係数として4 Log を加えて総 LRV が12あれば，最終的な原薬からウイルスが十分に除去されると考えられ，②の製造工程はレトロウイルスに対する安全性が確保されていると判断できる。

6.2. ウイルス力価の測定法

ウイルス力価（感染価）の測定法には定量法と半定量法がある。定量法には培養細胞を用いたプラーク法などがあり，1プラークが1感染単位（プラーク形成単位：PFU）に相当する。半定量法とは動物を用いた感染性試験や培養細胞を用いた $TCID_{50}$（50% Tissue Culture Infectious Dose; 50%細胞変性終末点）法において，感染の有無のスコアを基に定量する方法である。プラーク法と $TCID_{50}$法の操作の概略を以下に示す。

(1) プラーク法によるウイルス力価測定の操作例

①指標細胞の懸濁液を調製し，6穴培養プレートに播種して CO_2インキュベータでサブコンフルエントまで培養する。

②測定試料（ウイルス液）を培地で希釈し，10倍段階希釈液を調製する。

③培地を除去し，希釈したウイルス液 1 ml を各穴に入れ，数時間培養して感染させる。

④ウイルス液を除去し，寒天培地を入れて固まるまで放置する。

⑤数日間培養後，ニュートラルレッドを加えた染色用寒天培地を重層し，プラーク（図5）が観察されるまで6〜24時間培養する。

⑥培養後，各穴のプラークを数える。希釈倍数 10^k のプラーク数が N_1，N_2，N_3 の時（同一希釈が3穴の場合），以下の式によりウイルス感染価を求める。

$$感染性ウイルス\ (PFU/ml) = (N_1+N_2+N_3)\times10^k/3$$

(2) $TCID_{50}$法による力価測定の操作例

①指標細胞の懸濁液を調製し，96穴培養プレートに播種してサブコンフルエントまで培養する。

②測定試料（ウイルス液）を適度に希釈（図6の例では100倍希釈）し，新しいプレートの2列目に入れ，他の穴には希釈用無血清培地を入れる。

③8連ピペットを用いてプレート上で段階希釈列を作製する（図6の例では5倍希釈列）。

④細胞培養プレートから培地を除去し，ウイルス希釈列のプレートから細胞プレートに100 μl ずつ移し，数時間培養して感染させる。

⑤培地を添加し，数日間培養する。

⑥各穴の CPE の出現を顕微鏡観察により判定

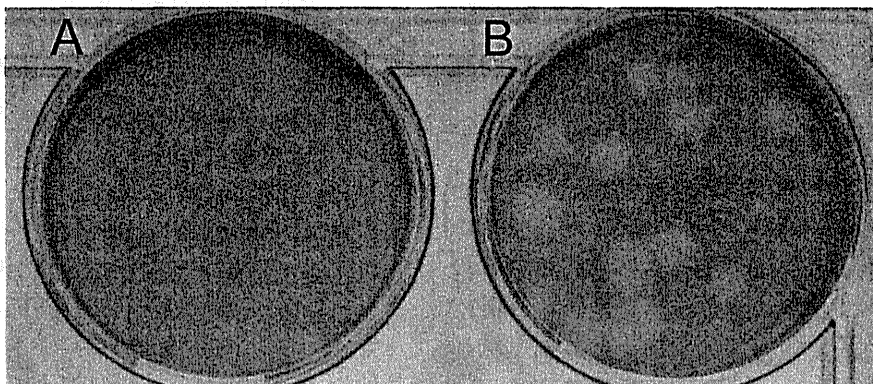

図5．ウイルスのプラーク
HAV を感染させた FRhK-4 細胞を感染5日目に固定，染色した。A：非感染細胞，B：ウイルス感染細胞。B ではウイルス感染による CPE が同心円状に広がり，白く抜けたプラークとして観察される。（写真提供：日本ケミカルリサーチ）

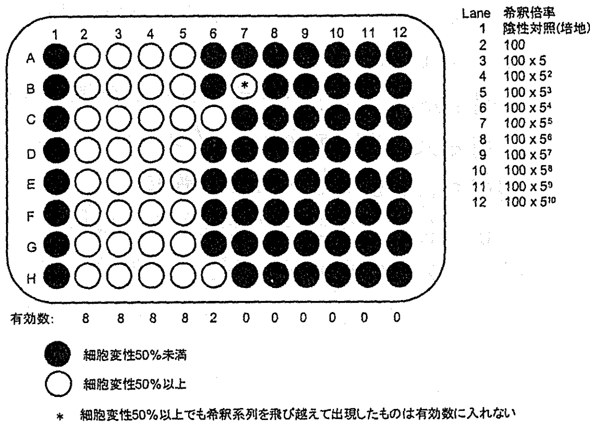

図6．$TCID_{50}$の実験例

し，記録する。

⑦以下のKarberの式を用いて$TCID_{50}$を計算する。

$TCID_{50}$=（2列目の希釈率）×（希釈率）$^{\Sigma-0.5}$

但し，

Σ＝（各希釈段階でのCPE数）/（各希釈段階の検体数）

図6の例では，

$\Sigma=8/8+8/8+8/8+8/8+2/8=4.25$

$TCID_{50}/ml=(10^2\times5^{(4.25-0.5)})/0.1\ (ml)=4.2\times10^5$

となる。$TCID_{50}$はPFUとほぼ一致するとされる。

(3) ウイルスクリアランスのNATによる定量法

感染性試験ではなくNATによりウイルス核酸量を力価に置き換えてクリアランス評価に用いる場合もある。NATを適用すると，感染性試験よりも迅速・高感度に測定が可能であり，また感染価測定と核酸検出法の組合せによりウイルスの除去と不活化を区別して測定できるという利点がある。一方，NATは測定試料中の感染性のない核酸断片を検出するため，ウイルス除去能を過小評価する可能性があることや，NATの阻害因子にも注意が必要など，特徴をよく知ったうえで利用することが必要である。NATを採用する場合には，十分なバリデーションが不可欠である。

おわりに

生物薬品のウイルス安全性確保は，さまざまな手段を相互補完的に組み合わせることにより達成され，また多様なウイルス試験が用いられている。紙面の関係もあり，ウイルス試験のすべてについて詳しく紹介することはできなかったが，興味のある方は他の総説等[5,6]も参考にしていただきたい。また，本稿で例示した試験法は必ずしも一般的，標準的な方法を示したものではなく，あくまで試験の概略をイメージするための一例として取り上げたものであり，実際には様々な方法が用いられていることをご理解いただければ幸いである。

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Collaborative Study to Establish a World Health Organization International Standard for Hepatitis E Virus RNA for Nucleic Acid Amplification Technology (NAT)-Based Assays

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Summary

The aim of the collaborative study was to evaluate candidate standards for hepatitis E virus (HEV) RNA for use in nucleic acid amplification technology (NAT)-based assays. The candidate standards consisted of lyophilized preparations of genotype 3a and genotype 3b HEV strains, obtained from blood donors, diluted in human plasma. The genotype 3a HEV strain has been developed as the candidate World Health Organization International Standard and the genotype 3b strain has been developed as the candidate Japanese National Standard. Coded duplicate samples of the two virus strains were distributed to the participating laboratories; genotype 3a HEV (Sample 1 and Sample 2) and genotype 3b HEV (Sample 3 and Sample 4). Each laboratory assayed the samples on 4 separate occasions and the data were collated and analyzed at the Paul-Ehrlich-Institut. Twenty-four laboratories from 10 countries participated in the study. Data were returned by twenty-three laboratories using both qualitative and quantitative assays. All assays were able to detect both candidate standards. It is proposed that the genotype 3a strain be established as the 1st International Standard for HEV RNA with a unitage of 250,000International Units per ml. On-going real-time and accelerated stability studies of the proposed International Standard are in progress.

Introduction

Hepatitis E virus (HEV) is a non-enveloped single stranded RNA virus belonging to the *Hepeviridae* family (Purcell and Emerson, 2008; Meng, 2010). In developing countries HEV is a major cause of acute hepatitis, transmitted by the faecal-oral route and associated with contamination of drinking water. In industrialized countries, HEV infection is being more frequently reported and whilst some cases are imported after travel to endemic areas, autochthonous cases are also increasing and infection with HEV appears more prevalent than originally believed (Ijaz *et al.*, 2009). Prospects for control of HEV infection are encouraged by recent efforts in vaccine development (Shrestha *et al.*, 2007; Zhu *et al.*, 2010). Four main genotypes, representing a single serotype, of HEV infect humans. Genotype 1 viruses are found mainly in Africa and Asia and genotype 2 in Africa and Central America. Genotype 3 and 4 viruses are generally less pathogenic, although some exceptions have been reported, particularly for genotype 4; these genotypes infect not only humans, but also animals including swine, wild boar and deer. While genotype 4 strains are restricted to parts of Asia, genotype 3 viruses are found throughout the world. Zoonotic transmission of HEV occurs, either by consumption of contaminated meat and meat products, or by contact with infected animals (Purcell and Emerson, 2010). An alternative route of transmission is by transfusion of blood components with reports from several different countries including, for example, the UK, France and Japan (Boxall *et al.*, 2006; Colson *et al.*, 2007; Matsubayashi *et al*, 2004; Matsubayashi *et al.*, 2008). Studies in Japan and China have identified acute HEV infections in blood donors confirmed by the detection of HEV RNA (Guo *et al.*, 2010; Sakata *et al.*, 2008).

It is now recognized that, in some countries at least, HEV infection is underreported, and where other causes of acute hepatitis have been excluded, HEV infection should be considered (Waar *et al.*, 2005). The diagnosis of HEV infection is based upon the detection of specific antibodies (IgM and IgG), however there are issues concerning the sensitivity and specificity of these assays (Bendall *et al.*, 2010; Drobeniuc *et al.*, 2010). Analysis of HEV RNA using nucleic acid amplification techniques (NAT) is also used for diagnosis and can identify active infection and help to confirm serological results (Huang *et al.*, 2010).

Infection with HEV may be particularly severe during pregnancy and in individuals with existing liver disease. Chronic HEV infection is an emerging problem amongst solid organ transplant recipients (Kamar *et al.*, 2008; Legrand-Abravanel *et al.*, 2010). In chronically infected patients, viral loads are monitored to investigate the efficacy of antiviral treatment (Haagsma *et al.*, 2010;

Kamar *et al*, 2010a; Kamar *et al.*, 2010b) and effects of reduction of immunosuppressive therapy (Kamar *et al.*, 2010c).
Several NAT assays have been reported for the detection of HEV RNA in serum and plasma or faecal samples, including conventional reverse transcriptase polymerase chain reaction (RT-PCR) as well as real-time RT-PCR, and reverse transcription-loop-mediated isothermal amplification (Lan *et al.*, 2009). The NAT tests include generic assays developed for the detection of HEV genotypes 1-4 (Jothikumar *et al.*, 2006; Gyarmati *et al.*, 2007). A recent study organized by the Paul-Ehrlich-Institut (PEI) on behalf of the World Health Organization (WHO), investigated the performance of HEV NAT assays in an international study (Baylis *et al.*, 2011). Dilution panels of different HEV strains were blinded and tested by laboratories with experience in detection of HEV RNA. The results of the study demonstrated wide variations in assay sensitivity (100-1000 fold, for the majority of assays). The proposal by the PEI to prepare a standard for HEV RNA for use in NAT-based assays was endorsed by the WHO Expert Committee on Biological standardization (ECBS) in 2009 (WHO/BS/09.2126) and following the initial study, two virus strains were selected for further development as a candidate International Standard for the WHO and a candidate Japanese National Standard in collaboration with the National Institute of Infectious Diseases (NIID) in Japan. The viral strains being developed as standards are genotype 3a and 3b HEV strains, which were equally well detected in the initial study and belong to genotype 3 which is widely distributed. The strains are both derived from blood donors with sufficient titres of HEV RNA to prepare standards of good potency. The aim of the present study is to establish the respective standards and demonstrate their suitability for use, evaluate the potency and assign an internationally agreed unitage.

Preparation of bulk materials

After the initial proficiency/strain evaluation study (Baylis *et al.*, 2011), two HEV strains were selected for the preparation of the candidate WHO International Standard and the candidate Japanese National Standard. The samples were kindly provided by Keiji Matsubayashi from the Japanese Red Cross Hokkaido Blood Center. The genotype 3a HEV strain HRC-HE104 was used to prepare the candidate WHO standard. The genotype 3b HEV strain JRC-HE3 was used to prepare the candidate Japanese National Standard. Characterization of the virus strains is shown in Table 1. The target concentration for the two bulk preparations was approximately 5.5 $\log_{10}$ HEV RNA copies/ml based upon the concentrations reported in the initial study (Baylis *et al.*, 2011) and the concentrations determined by the Japanese Red Cross Hokkaido Blood Centre. The two virus strains tested negative for HIV-1/2 RNA, HBV DNA and HCV RNA using the Cobas TaqScreen MPX test (Roche Molecular Systems Inc., Branchburg, USA).
For the preparation of the candidate WHO standard bulk, 131 ml of the HEV strain HRC-HE104 were mixed with 2015 ml of plasma. For the preparation of the candidate Japanese National Standard bulk, 30 ml of the HEV strain JRC-HE3 were mixed with 1070 ml of plasma. The bulk preparations were cooled (4-8°C) until processing (~18 hours later). The respective preparations were diluted using pooled citrated plasma which had been used in the initial HEV collaborative study (Baylis *et al.*, 2011). The plasma was centrifuged and filtered twice before use. The plasma diluent tested negative for anti-HEV IgG and IgM (Ulrich Mohn, Mikrogen GmbH, Neuried, Germany, personal communication) and tested negative for HEV RNA (data not shown) and HIV-1/2 RNA, HBV DNA and HCV RNA, testing was performed as described above. In addition, the plasma was negative for HBsAg, anti-HCV, anti-HBc and anti-HIV-1/2.
The filling and lyophilization was performed by an ISO 13485:2003 accredited Swiss company. For processing, 0.5 ml volumes were dispensed into 4 ml screw-cap glass vials. Rubber seals were then placed on top of the filled vials before loading into the freeze drier (CHRIST Epsilon 2-25 D) for lyophilization. After freeze-drying the vials were sealed with screw caps and vials stored at -20°C.

For the candidate WHO standard, 4256 vials were lyophilized; the coefficient of variation of the fill volume was 1.1%. In the case of the candidate Japanese National Standard, 2154 vials were lyophilized; the coefficient of variation of the fill volume was 1.0%. In both cases, measurements were made for a total of 26 vials. For analysis of residual moisture, vials filled with 0.5 ml volumes of plasma diluent were distributed throughout the freeze-drier. Residual moisture was 0.73%, as determined by testing of 12 vials (Karl Fischer analysis). The freeze-drying process did not affect the HEV RNA titre of the lyophilized samples when compared to aliquots of the respective bulk preparations which were stored at -80°C (data not shown).
Vials of the candidate WHO standard are held at the Paul-Ehrlich-Institut, Paul-Ehrlich-Straße 51-59, D-63225 Langen, Germany. The vials are kept at -20°C with continuous temperature monitoring.
All manufacturing records are held by PEI and are available on request by the ECBS.

Collaborative study

The collaborative study comprised 24 laboratories from 10 countries. The participants in the collaborative study who returned data are listed in Appendix 1.
The samples analysed in the study were labelled as Sample 1, Sample 2, Sample 3 and Sample 4. Sample 1 and Sample 2 were replicates of the candidate WHO standard; and Sample 3 and Sample 4 were replicates of the candidate Japanese National Standard. The collaborative study materials were shipped to participants at ambient temperature.
Participants were asked to test the panel using their routine assay for HEV RNA, testing the samples in four separate assay runs, using fresh vials of each sample for each run. Where laboratories performed quantitative tests, they were requested to report results in copies/ml, testing samples in the linear range of the assay. In the case of qualitative assays, participants were requested to assay each sample by a series of one $\log_{10}$ dilution steps, to obtain an initial estimate of an end-point. For the three subsequent assays, they were requested to assay half-$\log_{10}$ dilutions around the end-point estimated in their first assay. Participants reported diluting the materials using plasma, water or phosphate buffered saline. Data sheets and a method form were provided so that all relevant information could be recorded.

Statistical Methods

Quantitative Assays

Evaluation of quantitative assays was restricted to dilutions in the range between 0.0 $\log_{10}$ and -2.5 $\log_{10}$ where the assays of most participants seem to produce comparable data. For comparison of laboratories, the replicate results of each laboratory, corrected for the dilution factor, were combined as arithmetic mean of $\log_{10}$ copies/ml. Furthermore these estimates were combined to obtain an overall estimation for each sample by means of a mixed linear model with *laboratory* and *(log) dilution* as random factors.

Qualitative Assays

The data from all assays were pooled to give series of number positive out of number tested at each dilution. For each participant, these pooled results were evaluated by means of probit analysis to estimate the EC50 i.e. the concentration at which 50% of the samples tested were positive (for assays where the change from complete negative to complete positive results occurred in two or fewer dilution steps , the Spearman-Kaerber method was applied for EC50 estimation). The calculated end-point was used to give estimates expressed in $\log_{10}$ NAT-detectable units/ml after correcting for the equivalent volume of the test sample.

Relative potencies

Potencies of Samples 2, 3 and 4, for the quantitative assays, were estimated relative to Sample 1 using parallel line analysis of log transformed data. In the case of the qualitative assays, the relative potencies were determined using parallel line analysis of probit transformed data.

The statistical analysis was performed with SAS®/STAT software, version 9.2, SAS System for Windows. Estimation of end-point dilution and relative potencies were done with CombiStats Software, version 4.0, from EDQM/Council of Europe.

Stability studies

Stability of the candidate WHO standard is under continuous assessment, through both real-time and accelerated thermal degradation stability studies. Vials of the candidate WHO standard have been stored at -20°C (the normal storage temperature) and -80°C (to provide a baseline if there is any suggestion of instability at higher temperatures). For the accelerated thermal degradation, vials have been incubated at +4°C, +20°C, +37°C and +45°C for up to 4 months. After incubation at the respective temperatures, the contents of the vials were reconstituted in 0.5 ml of nuclease free water and analysed by real-time PCR (Jothikumar *et al.*, 2006).

Data Received

Data were received from a total of 23 participating laboratories; one laboratory failed to complete the study within the specified time frame. Data from 20 qualitative and 14 quantitative assays were reported. The types of assays used by participants are listed in Table 2; all assays were developed in-house. The assays used by participants were mainly based upon real-time PCR, although some conventional PCR methods were also used.
For the purposes of data analysis, each laboratory has been referred to by a code number allocated at random and not representing the order of listing in Appendix 1. Where a laboratory performed more than one assay method, the results from the different methods were analyzed independently, as if from separate laboratories, and coded, for example, laboratory 16a and laboratory 16b. In the case of 9 assays, quantitative values were reported covering the linear range of the respective assays; in addition, further dilutions have been performed allowing end-point determination. These data have been analysed separately and the number of estimates therefore exceeds the number of assay sets returned by the participants.

Results

Quantitative Assay Results

Initially evaluation of quantitative assays was performed without removing any outlying data; subsequently the data was restricted to a range between 0.0 $\log_{10}$ and -2.5 $\log_{10}$ where reproducible results were obtained across dilutions. The laboratory mean estimates in copies/ml ($\log_{10}$) are shown in histogram form in Figure 1. Each box represents the mean estimate from an individual laboratory, and is labelled with the laboratory code number. The individual laboratory means are given in Table 3. The relative variation of the individual laboratory estimates is illustrated by the box-and-whisker plots in Figure 2.

Qualitative Assay Results

The NAT-detectable units/ml ($\log_{10}$) for the qualitative assays are shown in histogram form in Figure 3. Each box represents the mean estimate from an individual laboratory and is labelled with the laboratory code number. The individual laboratory means are given in Table 4. From Figure 3, it can be seen that the estimates of NAT detectable units/ml ($\log_{10}$) from the qualitative

assays are more variable than the quantitative assays, reflecting the different sensitivities of the various assays. This observation is not unexpected and is in line with other studies.

Determination of Overall Laboratory Means

The overall means for the laboratories performing quantitative assays are shown in Table 5a. The means for both Sample 1 and Sample 2, replicates for the candidate WHO standard, are 5.58 $\log_{10}$ and 5.60 $\log_{10}$ copies/ml HEV RNA respectively, which demonstrates excellent agreement between the replicate samples. The candidate Japanese National Standard showed identical mean results of 5.66 $\log_{10}$ copies/ml HEV RNA for replicate Samples 3 and 4. The combined mean values for the replicate samples are shown in Table 5b.
The overall means for the qualitative assays are shown in Table 6a; there is good agreement between the duplicate samples as seen previously for the quantitative assays. The combined mean values for the replicate samples are shown in Table 6b. The qualitative assays show 0.3 $\log_{10}$ lower mean estimates than the quantitative assays.

Relative Potencies

Based upon the data from both qualitative and quantitative assays, the candidate WHO standard was estimated to have a potency of 5.39 $\log_{10}$ units/ml (95% confidence limits 5.15 – 5.63). This value was estimated with a combined end-point evaluation of qualitative and quantitative (restricted to dilutions in the range of 0.0 $\log_{10}$ to - 2.5 $\log_{10}$) data by means of a mixed linear model.
The potencies of Samples 2, 3 and 4 were calculated relative to Sample 1, taking the value of Sample 1 as 5.39 $\log_{10}$ units/ml. The relative potencies are shown in Tables 7 and 8 for the quantitative and qualitative assays, respectively. For the quantitative data from laboratory 9, no potency was estimable since there was only one dilution tested for each sample. The data is plotted in histogram form (Figures 4-6). The data demonstrate that expressing the results as potencies relative to Sample 1, as a standard with an assumed unitage of 5.39 $\log_{10}$ units/ml gives a marked improvement in the agreement between the majority of methods and laboratories. These data provide some evidence for commutability of the candidate standard for evaluation of HEV from infected individuals, since Samples 1 and 2 represent a different strain of HEV compared to Samples 3 and 4.

Results of Stability Studies

Vials of the candidate WHO standard were incubated at +4°C, +20°C, +37°C and +45°C for up to four months and tested by real-time PCR for HEV RNA. The heat-treated vials were assayed concurrently with vials that had been stored at -20°C and at -80°C. All samples were tested in duplicate and were compared to a standard curve prepared using vials of the candidate WHO standard stored at -80°C.
There was no evidence of instability of the samples stored at -20°C when compared to samples stored at -80°C. After 4 months incubation at +20°C a small loss of titre was observed.The observed drop in titre at higher temperatures (+37°C and +45°C) may be related to problems with reconstitution of the samples rather that actual degradation and has previously been observed for some other preparations, particularly for RNA viruses formulated in pooled plasma. The potency of the reconstituted material, after freezing and thawing, has not been investigated. Further stability studies (both real-time and accelerated) are on-going and will be communicated to the WHO.

All raw data for the collaborative study and stability analysis are held by PEI and are available on request by the ECBS.

Conclusions

In this study, a wide range of quantitative and qualitative assays were used to determine the suitability and evaluate the HEV RNA content of the candidate standards. Although the methods used by the study participants were all developed in-house, the majority of assays were able to detect the two HEV strains consistently. Based upon the data from the qualitative and the quantitative assays, the candidate WHO standard was estimated to have a potency of 5.39 $\log_{10}$ units/ml. Since the unitage assigned to the 1st WHO standard of a preparation is essentially arbitrary, for practical purposes, the candidate International Standard has been assigned a unitage of 250,000 International Units/ml. Since there was only a negligible difference in the overall means for the candidate Japanese National Standard compared to the WHO preparation, the two materials have therefore been assigned the same value i.e. 250,000 International Units/ml. In the case of the quantitative assays, laboratories reported values in HEV RNA copies/ml. The participants used plasmid DNA containing HEV sequences, synthetic oligonucleotides and *in vitro* transcribed HEV RNA to control for copy number. In some cases laboratories used HEV-containing plasma which had been calibrated against *in vitro* transcribed HEV RNA. Another laboratory prepared standard using stool-derived virus, the titre of which was determined by end-point dilution and analysis by Poisson distribution. No standard method or common quantitation standard material was used, and this is reflected in the variation observed for the quantitative results, with a variation in the order of 2 $\log_{10}$, which were improved by expressing the results against Sample 1 as a common standard. In the case of the qualitative assays, the variation in NAT-detectable units was at least 3 $\log_{10}$, and again expressing potencies relative to Sample 1 improved the agreement between the different laboratories and methods.
The collaborative study materials have been dispatched at ambient temperature, replicating the intended shipping conditions. Initial accelerated thermal degradation analysis indicates a reduction in the levels of HEV RNA at higher incubation temperatures. On-going studies on the real-time stability under normal storage conditions as well as studies concerning thermal degradation are in progress.
The standard will be of value for comparison of results between laboratories, determination of assay sensitivities and for validation. It is anticipated that the standard will find application in clinical laboratories, particularly hepatitis reference laboratories performing diagnosis and monitoring HEV viral loads in chronically infected transplant patients, research laboratories, blood and plasma centres which implement HEV NAT screening, regulatory agencies and organizations developing HEV vaccines as well as manufacturers of diagnostic kits.

Each vial of the HEV RNA standard contains the lyophilized residue of 0.5 ml of HEV RNA positive plasma. Predictions of stability indicate that the standard is stable and suitable for long-term use when stored as directed in the accompanying proposed "Instructions For Use" data sheets for the panel (Appendix 2).

Recommendations

Based upon the results of the collaborative study, it is proposed that the genotype 3a HEV strain (Samples 1 and 2, in this study) should be established as the 1st International Standard for hepatitis E virus RNA and be assigned a unitage of 250,000 International Units/ml. The standard has been given the code number 6329/10; 3800 vials are available to the WHO and custodian laboratory is the Paul-Ehrlich-Institut.

Comments from participants

After circulation of the draft report for comment, replies were received from all participants. The majority of the comments were editorial in nature and the report has been amended accordingly. All participants were in agreement with the conclusions of the report.

One participant commented on the possible incorrect estimation of the viral load by the participants who used DNA standards (synthetic oligonucleotides or plasmid DNA) due to lack of control for reverse transcription of virus RNA into cDNA. This might be better controlled using *in vitro* transcribed RNA or a virion-based preparation.

Another participant remarked that many laboratories have used the same method, showing quite different sensitivities, possibly due to differences in extraction and amplification/detection reagents and instrumentation and its set up.

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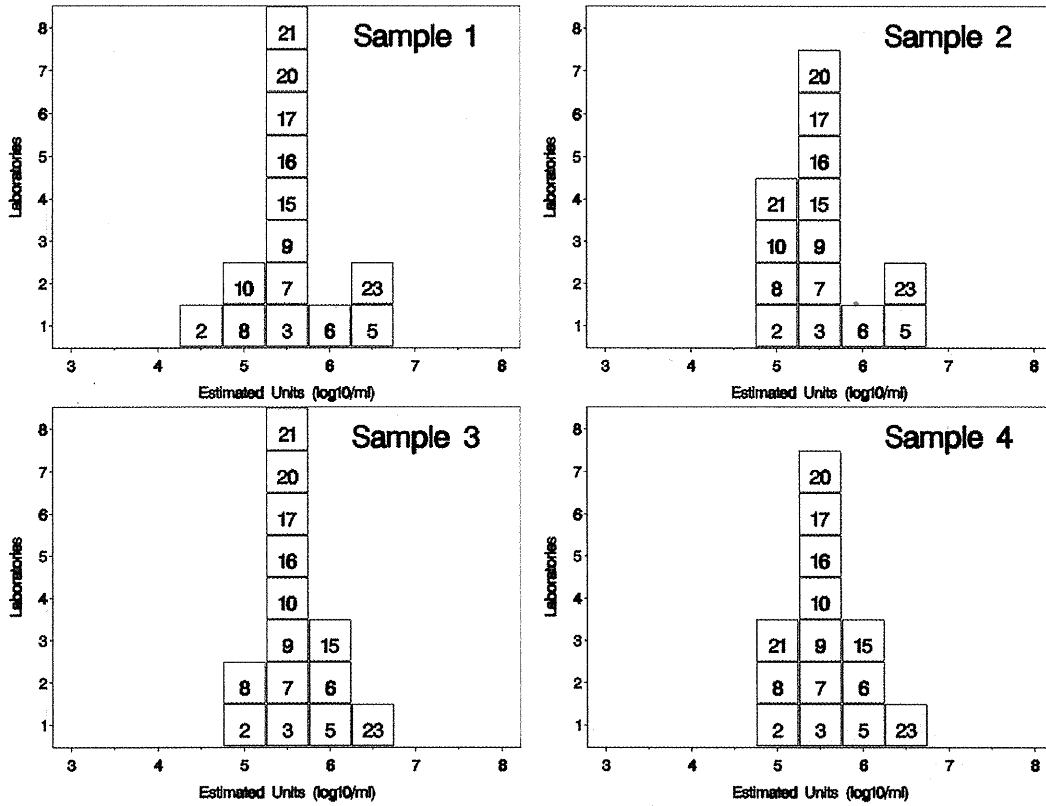
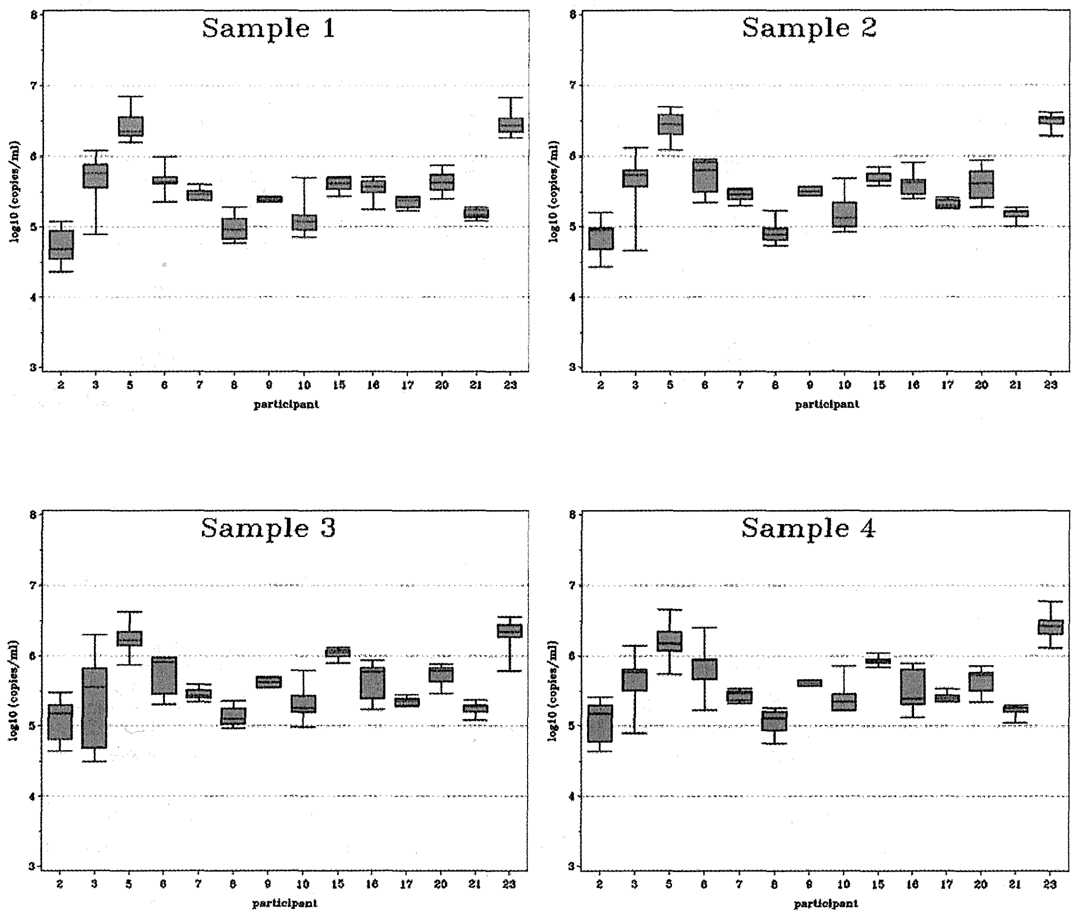
Figure 1 Estimates for quantitative assays



Histograms of the quantitative results for participating laboratories for Sample 1, Sample 2, Sample 3 and Sample 4. Estimates of $\log_{10}$ copies/ml are indicated on the x-axis. Data are shown for laboratory 16a.

Figure 2 Box and whisker plots of the quantitative data (log_{10} copies/ml)



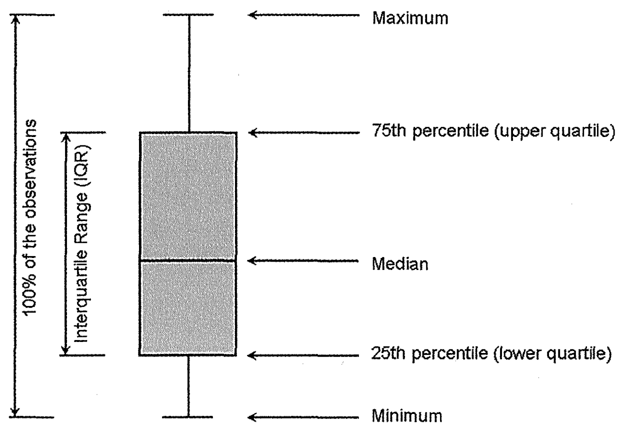
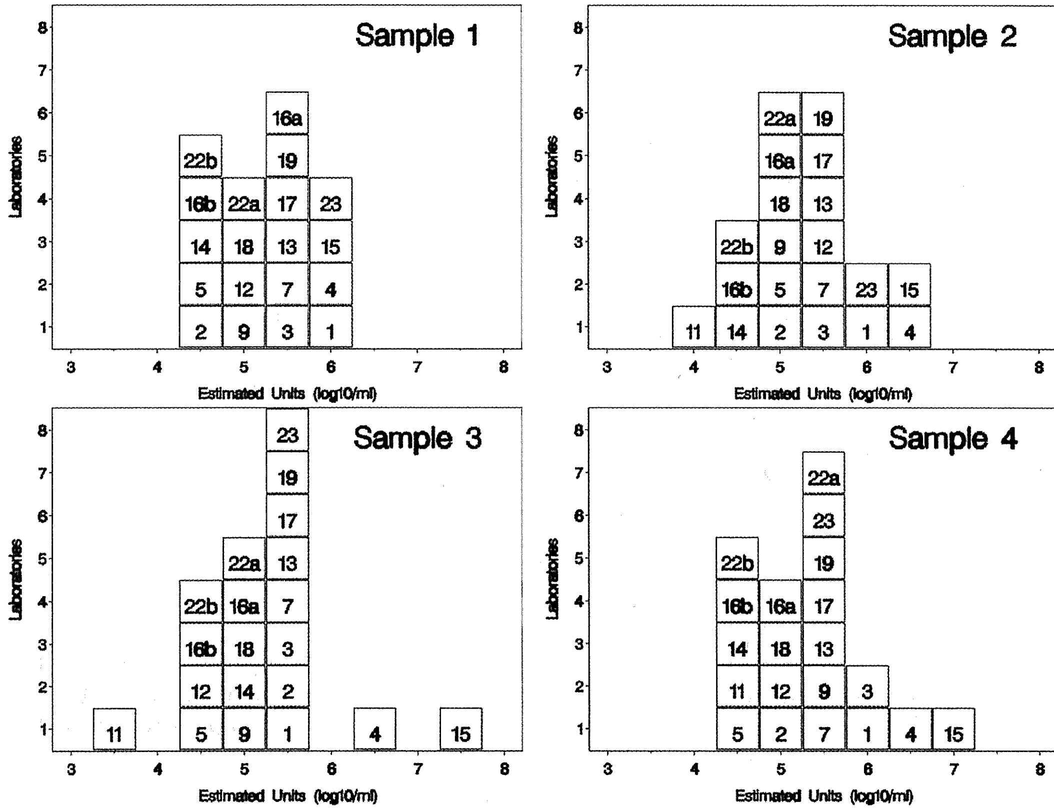
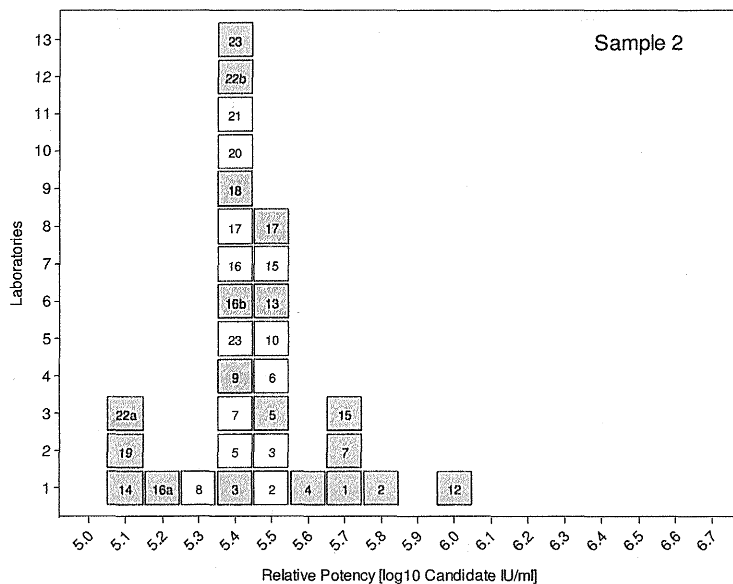


Figure 3 Estimates for qualitative assays



Histograms of the qualitative results for participating laboratories for Sample 1, Sample 2, Sample 3 and Sample 4. Estimates of $\log_{10}$ NAT-detectable units/ml are indicated on the x-axis. In the case of laboratory 11, data for Sample 1 have been omitted due to a 2 $\log_{10}$ higher cut-off.

Figure 4 Potency of Sample 2 relative to Sample 1



Histogram of the potency of Sample 2 relative to Sample 1 (=5.39 log_{10} units/ml); qualitative data (grey boxes) and quantitative data (white boxes). No relative potency is shown for laboratory 11 for sample 2, since no value had been determined for Sample 1 (i.e. the data were outlying and did not perform as the replicate i.e. Sample 2).