紹　介

―アジア地域臨床獣医師等総合研修事業における研修報告等（Ⅰ）―

1　研修報告等掲載の経緯

平成 28 年度から本会で取り組んでいます「アジア地域臨床獣医師等総合研修事業」において，獣医学系大学における研修による新たな技術・知見の修得の成果指標として，獣医学術雑誌等への論文または報告書の掲載が義務付けられています．この要件に従い，研修生から研修終了後に提出される報告書等については，事業推進に資するため，日本獣医師会雑誌（会誌部分）に投稿・掲載することとしました．しかし，研修生本人の執筆による報告書等は英文であるため，日本獣医師会雑誌投稿規程を一部改正し，英文による投稿及び掲載ができることとしました．なお，Abstract（要約）及び英文の概要について，和訳を追加掲載します．

2　確認事項

①本稿は，本会が実施する「アジア地域臨床獣医師等総合研修事業」に参加したアジア各国の研修生に義務付けられた報告書であり，学術論文には該当しません．

②他の学術誌等に学術論文等として掲載されたものについては，本欄においてその旨を紹介し，報告に代えます．

③研修生及びその指導教員に，研修報告書を学術論文として投稿しないことをあらかじめ確認し，学術論文として他の学術誌への投稿希望がある場合は，本誌には掲載せず，②に示すとおり紹介だけ行います．

Comparison of type and concentration of overlay media which effect on cell confluency and incubation period on plaque formation in viral plaque assay

ウイルスのプラックアッセイにおけるプラック形成時の細胞増殖性とその培養期間に影響を及ぼす重層液の種類と濃度の比較

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Abstract

Plaque assay is one of the accurate methods for direct quantification of tissue culture adaptive infectious virus by simply counting the discrete plaques in a cell culture. Even it is a traditional method; plaque assay is still regarded as the gold standard for virus quantification. In this study, I tried to examine the effect of overlay media, cell confluency and incubation period on discrete plaque formation for Bovine Herpes Virus-1 (BHV-1) LA strain on MDBK cell culture. The data provides the true values for the plaque assay protocol for BHV-1.

—— Key words : Bovine Herpes Virus-1, MDBK cells, plaque assay, virus quantification.

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要　　約

プラックアッセイは，培養細胞系で形成されたプラックを数えることで，容易に細胞に順化したウイルスを直接定量できる正確な手法である．プラックアッセイは，従来使われている手法ではあるが，ウイルスの定量法として依然として標準法（ゴールドスタンダード）の１つとみなされている．この研究では，牛ヘルペスウイルス１型 LA 株（BHV-1 LA）のプラック形成に影響する重層液の種類，濃度，培養時間の条件について MDBK 細胞を用いて調べた．本研究で得られた結果は BHV-1 型のプラックアッセイの正確性に寄与できる．

――キーワード：牛ヘルペスウイルス１型，MDBK 細胞，プラックアッセイ，ウイルス定量．

1. Introduction
（緒　　言）

Viral plaque assay is the method for measuring viral infectivity and multiplication in cultured cells. Clear lysed areas or plaques develop as the viral particles are released from the infected cells during incubation [1].

（プラックアッセイは培養細胞を用いてウイルスの感染価を計る方法である．）

Plaque assay is one of the most accurate methods for the direct quantification of infectious virons and antiviral substances through the counting of discrete plaques [2]. This technique was first developed to calculate the titer of bacteriophage stocks. Renato Dulbecco modified this procedure in 1952 for use in animal virology and it has since been used for reliable determination of the titers of many different viruses [3]. It is widely regarded as the gold standard for virus quantification and heavily used in the field of virology [4]. Virus plaque assay only can be used for the viruses which can cause cytopathic effect in host cells [5]. Therefore, the usage is limited but the main advantages of plaque assay are that each individual virus (or aggregate) forms a single plaque and that each plaque is rarely a mixture of several virus types [6].

（本法は形成されたプラック数を数えることで直接定量的に感染性ウイルス粒子数を正確に計測でき，また抗ウイルス効果を調べる方法としても利用される．本手法はウイルス学の分野においてウイルスの定量法として重用され，宿主細胞で細胞変性が認められるウイルスに対してのみ利用可能である．）

In this procedure a confluent monolayer of host cells is infected with a lytic virus of an unknown concentration and allows absorbing. Unabsorbed virus is washed off and overlay medium is added to restrict the spread of progeny virus to surrounding cells. Typically solid or semisolid overlay substrates like Agarose, Methyl Cellulose and Carboxymethyl Cellulose have been used [2]. Following visible plaque formation cells are fixed and stained to identify plaques by naked eye. The number of plaques; Plaque Forming Units (PFU) and the dilution of the virus solution is used to calculate the titer of virus [3].

（手法としては，単層培養させた宿主細胞に濃度が不明のウイルス液を感染させて吸着させる．吸着後ウイルス液を洗い流してウイルスの拡散防止の目的で細胞に重層液を加える．通常アガロースやメチルセルロース，カルボオキシセルロースなどの固相，半固相の物質が重層液に加えられている．その後プラックを肉眼で可視化するため，感染細胞は固定染色される．

プラック形成単位（力価：Plaque Forming Unit）はプラック数と希釈倍率を用いることで以下の式で算出される．）

Virus titer = PFU / dilution of the virus inoculum

（ウイルス力価（PFU）＝プラック形成数／接種ウイルスの希釈倍率）

In this report, to know the most optimum condition of viral plaque assay for Bovine Herpes virus-1 (BHV-1) LA strain as the tissue adapted virus, I examined the effect following factors on distinguish plaque formation.

（本研究では細胞に順化した牛ウイルス１型（BHV-1）LA 株を用いてプラックアッセイの最適条件を求めるために明瞭なプラック形成にかかわる下記の因子の影響を調べた．）

1. Effect of solid and semisolid overlay media
2. Effect of concentration of overlay media
3. Effect of cell confluent of monolayer cells
4. Effect of time duration between virus inoculation and fixation

（①重層液の固相，半固相の影響
②重層液の影響
③単層培養の濃度の影響
④ウイルス培養から固定までの培養時間の影響）

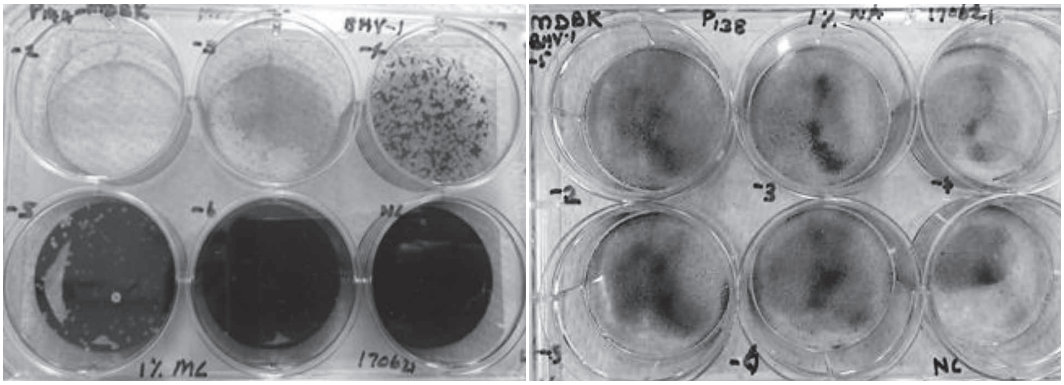


Fig 1（図 1）

2. Materials and methods
（材料と方法）

2.1 Effect of solid (Nobel agar) and semisolid (Methyl Cellulose) media on plaque formation

（プラック形成に与える固相（ノーブルアガー）と半固相（メチルセルロース）の影響）

Four C6 plates of MDBK cells of ＞90% of cell confluent were prepared and each were inoculated with 10 fold serial dilutions of BHV-1 (10^{-2} to 10^{-6}) and kept in 5% CO_2 incubator at 37°C for 1 hour for virus absorption. Following absorption, the virus solution was aspirated out and each of 0.75% Nobel agar, 1% Nobel agar, 0.75% Methyl cellulose and 1% Methyl cellulose overlay media were added onto the plates, respectively. C6 plates were incubated in 5% CO_2 incubator at 37°C for three to five days. Then, cells were fixed with 10% formaldehyde/PBS and stained using 0.5% crystal violet.

2.2 Effect of cell confluence on distinguish plaque formation

（明瞭なプラック形成に対する細胞濃度の影響）

Three C6 plates of MDBK cells with 80%, 90% and ＞100% confluent were prepared. To obtained ＞100% confluent kept the plates one more day following it became 100% confluent. Ten-fold diluted BHV-1 (10^{-2} to 10^{-6}) were prepared and inoculated into cultured cells. Following viral absorption in 5% CO_2 incubator at 37°C for 1 hour, 1.0% of Methyl Cellulose overlay medium was added for each plate and continued the protocol as mentioned above. PFU was calculated in each plate.

2.3 Effect of concentration of methyl cellulose overlay media on distinguish plaque formation

（明瞭なプラック形成に対するメチルセルロースを重層液の濃度の影響）

Ten-fold diluted BHV-1 (10^{-2} to 10^{-6}) were inoculated onto three C6 plates of MDBK cells which were ＞90% confluent. Following viral absorption, 0.75%, 1.0% and 1.25% methyl cellulose overlay medium were added to the well of C6 plates, respectively. Plates were incubated for three days in 5% CO_2 incubator, then fixed and stained as mentioned above. C6 plates were examined the size and shape of the plaques.

2.4 Effect of incubation time after virus inoculation

（ウイルス吸着後の培養時間の影響）

Three C6 plates of MDBK cells which were 90% confluent were prepared and were inoculated with ten-fold dilutions of BHV-1 (10^{-2} to 10^{-6}). Following viral absorption 0.75% of Methyl cellulose overlay medium was added. Each C6 plate was fixed on day 3, day 4 and day 5, respectively, after virus inoculation and PFU was examined.

3. Results
（結　　果）

3.1 Effect of solid (Nobel agar) and semisolid (Methyl Cellulose) media on plaque formation

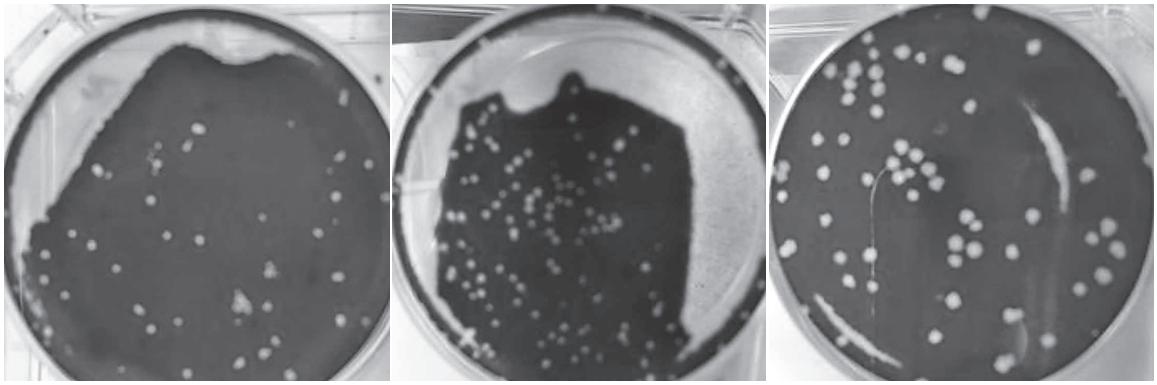
（プラック形成に与える固相（ノーブルアガー）と半固相（メチルセルロース）の影響）

Observed clear plaque formation only with Methyl Cellulose overlay medium (Fig 1).

（メチルセルロースを重層液としたプレートにおいて明瞭なプラック形成が観察された（図 1）.）

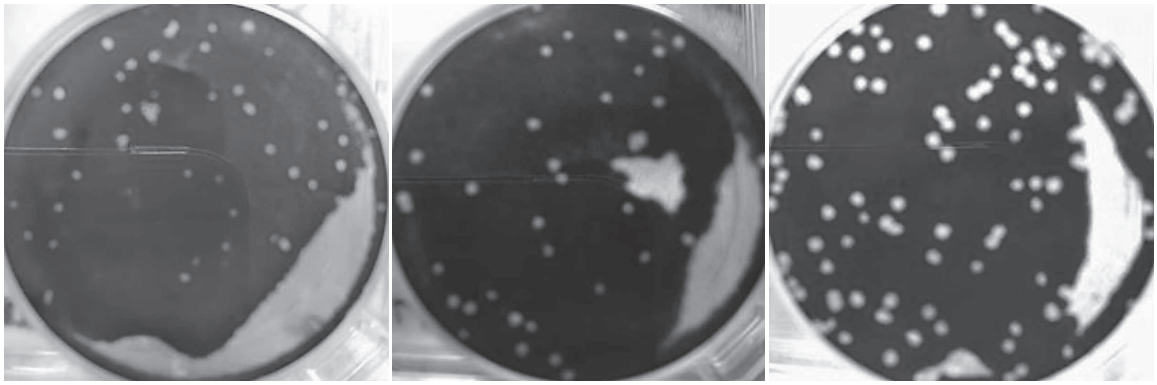
Table X. Virus plaque formation units (PFU) in the presence of various concentration of methyl cellulose
表X　各種濃度のメチルセルロースにおけるウイルスプラック形成単位（PFU）

Cell confluency 細胞濃度(占有率)	Concentration of Methyl Cellulose overlay media 重層液中のメチルセルロースの濃度		
	0.75%	1.0%	1.25%
80%	1.33×10^7	1.05×10^7	1.3×10^7
90-100%	3.3×10^7	3.5×10^7	3.2×10^7
＞100%（100%以上）	2.08×10^7	1.28×10^7	1.88×10^7



1.25%　1.0%　0.75%

Fig 2（図 2）



3 days after　4 days after　5 days after

Fig 3（図 3）

3.2 Effect of cell confluence on distinguish plaque formation
（明瞭なプラック形成に対する細胞濃度の影響）

The result showed that high virus titer when the host cells were 90-100% confluent (Table X). However, cell confluency was not associated with PFU.

（宿主細胞が90～100%の濃度で最も高いウイルス力価を得られた（表X）．しかしながら，細胞の濃度はPFUには関与していなかった．）

3.3 Effect of concentration of Methyl Cellulose on plaque formation
（明瞭なプラック形成に対するメチルセルロースを重層液の濃度の影響）

0.75% of Methyl Cellulose overlay medium provided comparatively bigger plaques than the 1% and 1.25% Methyl Cellulose (Fig 2).

（0.75%メチルセルロース含有重層液において，他の1.0%，1.25%含有液よりかなり大きなプラックの形成が認められた（図2）．）

3.4 Effect of incubation time after virus inoculation
（ウイルス吸着後の培養時間の影響）

Cells fixed five days after inoculation showed distinguish large plaques compare to cells fixed on three days and four days after virus inoculation (Fig 3).

（培養後5日目に固定したプレートにおいて3日目，4日目培養に比べて明瞭な大きなプラックが形成された（図3）.

4. Discussion
（考　　察）

Formation of plaque in a virus plaque assay is due to cytopathic effect made by the virus strain. Cytopathic effect on cell layers with Methyl cellulose medium was observed. On the other hand, cells were detached from the bottom of plates in case of Novel agar and following fixation and staining, cells had been washed off from the plates. Procedure was repeated by changing the temperature of novel agar between 40°C and 45°C and incubating the plates in upside down position in 5% CO_2 incubator. The results were same. Although the reason was unknown, it may indicate solid Nobel agar disturbed the plaque formation by BHV-1 while semi solid Methyl cellulose gives distinguish plaque with BHV-1.

（ウイルスプラックアッセイにおけるプラックの形成はウイルスによる細胞変性効果によって発現する．単層培養細胞における細胞変性効果はメチルセルロース含有培地を用いて観察された．一方，ノーブルアガーを用いた場合には，細胞がプレートの底面から剝離して固定染色後にはプレートから洗い流された．ノーブルアガーを用いた場合にはBHV-1によるプラックの形成は阻害されるのに対して，半固相のメチルセルロースを重層した場合には明瞭なプラックが観察された.

Effect of cell confluency in plaque formation was measured by using different % confluent of MDBK cells. The initial concentration of cells per one well of C6 plate was maintained from 4×10^5 to 4.3×10^5. Cell confluent of 90-100% showed high PFU compare to 80% and >100%. When overlay media is applied following initial viral infection, plaques will begin to develop as viral infection and replication are restricted to the neighboring cells. As the cells are loosely attached in cell layers of 80% confluent cells, discrete plaque formation could not be observed. At the same time when the cells are kept for a longer duration and cell confluent becomes >100%, the monolayer structure get changed and it disturbs the formation of discrete plaques during the given incubation period. Therefore 90–100% confluent of cells is best for the viral plaque assay.

（プラック形成における培養細胞の密度の影響はMDBK細胞の異なった濃度（%）で測定を行った．培養細胞の密度が90～100%の場合に，その密度が80%や100%以上の場合に比べて高いプラック形成単位を示した.

The concentration of Methyl Cellulose as overlay media has inversely affected the size of the plaques. Although PFU number is more or less similar with all three concentrations, the diameter of single plaque with 0.75% Methyl cellulose is slightly higher than with 1.0% or 1.25%. When the concentration of overlay medium is low it positively effects on replication-lysis-infection cycle of the virus and propagation of infection to surrounding monolayer.

（重層液中のメチルセルロース含量はプラックの大きさに反比例的に影響した．これは，メチルセルロースの含有濃度が低い方が，単層培養細胞ではウイルスの増殖，細胞の融解，感染のサイクルや感染の広がりに有効に働くためである.

Time duration following inoculation of virus to fixation provided similar PFU in all three plates. However, clear distinguish comparatively large plaques were visible in the plate fixed on five days after virus inoculation. Five days culture after virus inoculation may be optimal for BHV-1 plaque assay.

（固定までの3種類の異なる培養期間ではそのプラック形成単位はいずれも変わらなかった．しかしながら，5日間培養したプレートにおいて明瞭な比較的大きなプラックが観察された．BHV-1のプラックアッセイにおいては5日間の培養が最適であると考えられた.

Finally, these results in this study provide the true value on the plaque assay protocol for BHV-1.

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