

马病毒性动脉炎中和试验

Serum Neutralization Test for Infection with equine arteritis virus

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11-30-2021

马病毒性动脉炎

Infection with equine arteritis virus

- 马病毒性动脉炎 (EVA) 是一种经济上重要的马属动物病毒病。种马可以成为病毒的长期携带者，并在繁殖过程中传播病毒。一些马也会出现急性疾病。尽管成年马的死亡非常罕见，但感染怀孕母马可能会流产，而幼龄马驹可能会死于性肺炎和肠炎
- Infection with equine arteritis virus (EVA) is an economically important viral disease of equids. Stallions can become long term carriers of the virus, and transmit it during breeding. Acute illness also occurs in some horses. Although deaths are very rare in healthy adults, infected pregnant mares may abort, and very young foals may die of pneumonia and enteritis.

法定报告疫病

Notifiable disease

- OIE名录中须通报疫病
- **Notifiable disease in OIE list**
- 世界各国须报告疫病
- **Reportable disease in many countries**
- 《中华人民共和国进境动物检疫疫病名录》二类病
- **List B of Quarantine diseases for animals imported to the People' Republic of China**

地理分布

Geographic Distribution

- 大多数国家/地区都发现了EAV抗体。在北美和南美、欧洲、亚洲、非洲和澳大利亚已经报道了血清阳性马。
- 冰岛和日本没有
- 中国没有发现
- Antibodies to EAV found in most countries in North and South America, Europe, Asia, Africa and Australia.
- Absent from Iceland and Japan
- Not found in China

病原学

Etiology

- 马动脉炎病毒：动脉炎病毒科动脉炎病毒属
- Equine arteritis virus(EAV) in the genus Arterivirus, family Arteriviridae
- 有囊膜的正股单链RNA病毒
- enveloped, positive-sense, single-stranded RNA virus
- 基因组12.kb, 至少10个开放阅读框 (ORF)
- Viral genome 12.7 kb, at least 10 open reading frames (ORF).

理化特性

Physical and chemical characteristics

- 病毒在 56-58°C 下在 20-30 分钟内灭活，但在 37-38°C 下存活 2-3 天，在 4-8°C 下存活长达 75 天。
- This virus is inactivated in 20–30 min at 56-58°C, but can remain viable for 2 -3 days at 37-38°C and for up to 75 days at 4-8°C.
- 病毒对乙醚、氯仿等脂溶剂以及普通消毒剂和去污剂敏感。
- EAV is readily inactivated by such as ether and chloroform. common disinfectants and detergents
- 病毒在低温条件下极稳定，在 -20°C 保存 7 年仍有活性。
- The virus is extremely stable under low temperature and is still live when stored at -20°C for 7 years.
- 精液冷冻后仍具有传染性。
- Semen remains infectious after freezing.

血清型

Serotype

- 只有一个血清型，原型毒株Bucyrus
- Only one serotype, prototype Bucyrus strain
- 世界各地 EAV 分离株分为北美 (NA) 和欧洲 (EU) 谱系，并进一步细分为两个北美 (NA-1 和 NA-2) 和两个欧洲 (EU-1 和 EU-2) 进化枝。
- EAV isolates from around the world: North American (NA) and European (EU) lineages, further subdivided into two North American (NA-1 and NA-2) and European (EU-1 and EU-2) clades.
- 时间和地理上不同的病毒野毒株之间存在基因组和抗原变异，以及毒力、致病性和中和表型方面存在差异。
- Genomic and antigenic variation, and difference in their virulence, pathogenicity and neutralization phenotype exists among temporally and geographically different strains of EAV field virus.
- 一些野外菌株能够引起中度至重度的临床症状，而另一些仅引起轻微的临床疾病。
- Some field strains of EAV are capable of causing moderate to severe clinical signs whereas others only induce mild clinical disease.

易感动物种类

Species Affected

- 马属动物 (Equidae)
 - --马 (Horse)
 - --矮马 (Pony)
 - --驴 (Donkey)
 - --斑马 (Zebra)
- 南美骆驼 (South American Camelid)
- **公共卫生 : Public Health**
- **人感染报道 : 无**
- No report of infecting humans.

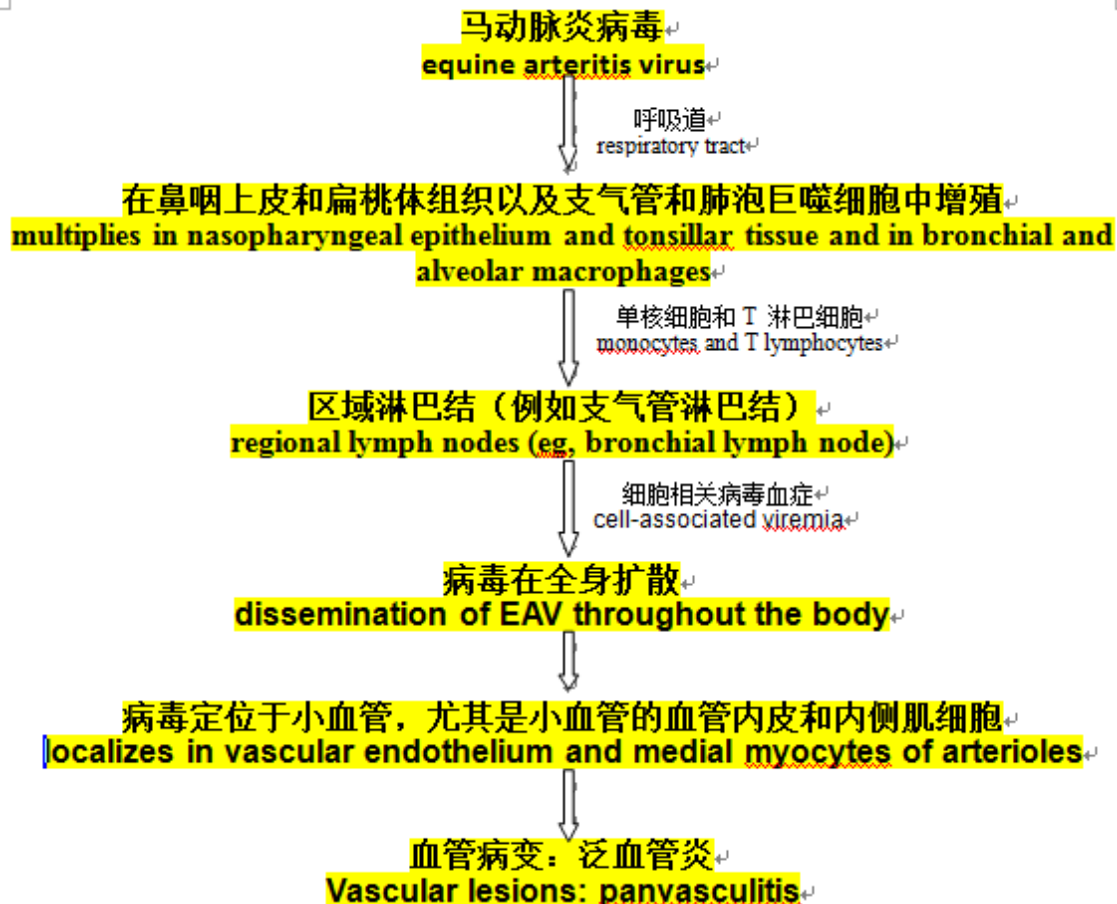
传播途径

Route of transmission

- 马动脉炎病毒可以通过呼吸道和性途径传播。
- 病毒可垂直传播给胎儿
- 病毒存在于呼吸道分泌物、尿液、粪便、生殖道；
- 母马在感染后的短时间内，EAV 在阴道和子宫分泌物以及卵巢和输卵管中发现。
- 种马在精液中排出 EAV，并携带病毒多年。一些携带者可能最终会清除感染。
- 母马、阉马或性不成熟的小马尚未报告真正的携带者状态。
- Infection with equine arteritis virus can be transmitted by the respiratory and the venereal routes.
- EAV is vertically transmitted to the fetus.
- Virus found in respiratory secretions, urine and feces and in the reproductive tract
- In mares, EAV can be found in vaginal, uterine secretions, and in the ovary and oviduct, for a short period after infection.
- Stallions shed EAV in semen, and can carry the virus for years. Some carriers may eventually clear the infection.
- True carrier states have not been reported in mares, geldings or sexually immature colts.

致病机理

Pathogenesis



病理变化

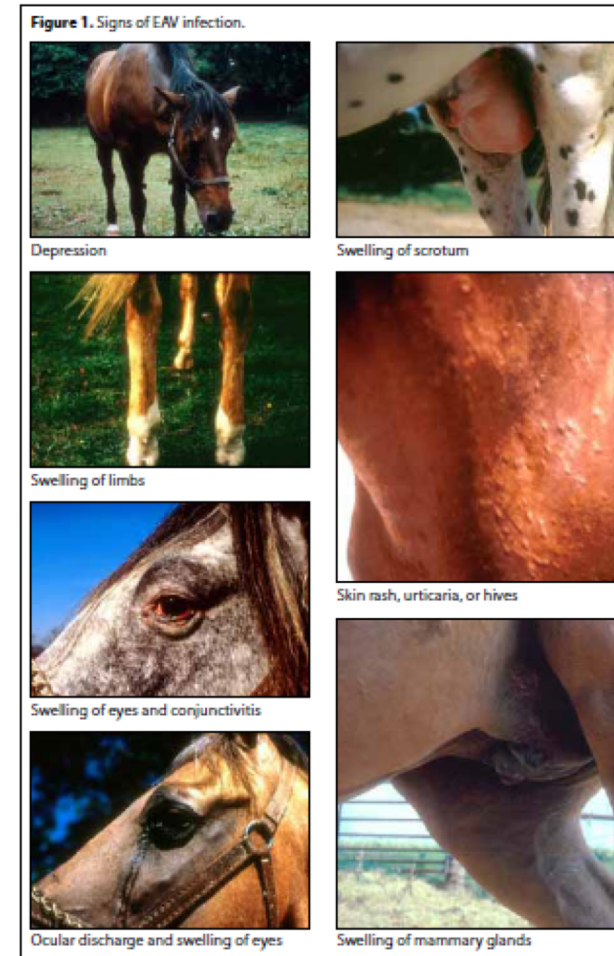
Lesions

- **最显着的肉眼发现：**水肿、充血和出血，尤其是在四肢和腹部的皮下组织；腹内和胸腔淋巴结以及小肠和大肠的水肿和出血。
- 小马驹会出现肺水肿、肺气肿和间质性肺炎、肠炎和脾脏梗塞。
- **特征性微观病变**是血管炎，主要小动脉和小静脉。
- 组织学变化:轻度病例的血管和血管周围水肿；严重病例的中膜纤维蛋白样坏死、广泛淋巴细胞浸润、内皮坏死和缺失以及血栓形成。
- Most significant **gross findings: edema, congestion, and hemorrhages**, especially in the subcutis of the limbs and abdomen; and edema and hemorrhage of the intra-abdominal and thoracic lymph nodes and of the small and large intestine
- Pulmonary edema, emphysema and interstitial pneumonia, enteritis, and infarcts in the spleen in foals
- **Characteristic microscopic: vasculitis**, involving primarily smaller arterioles and venules.
- Histological changes : vascular and perivascular edema in mild cases; fibrinoid necrosis of tunica media, extensive lymphocytic infiltration, necrosis and loss of endothelium, and thrombus formation in severe cases.

临床症状

Clinical Signs

- • 发烧、食欲不振、沉郁、淋巴细胞减少
- • fever, loss of appetite, depression, lymphocytopenia
- • 肢体、阴囊、鞘和乳腺水肿或肿胀
- • edema or swelling of limbs, scrotum, sheath, and mammary glands
- • 呼吸道症状，包括鼻腔
- • respiratory signs, including nasal
- • 结膜炎或“红眼病”或眼部分泌物
- • conjunctivitis or "pink-eye" or ocular discharge
- • 皮疹• skin rash
- • 怀孕母马流产
- • abortion in the pregnant mare
- • 小马驹间质性肺炎和肠炎
- • interstitial pneumonia and enteritis in young foals



治疗

Treatment

- 大部分感染马可完全恢复
- *Affected horses almost make clinical recoveries*
- 无特性的抗病毒治疗药物
- no specific antiviral treatment
- 支持性治疗：中度至重度发病马
- Supportive treatment: moderate to severely affected horses
 - • 退热药antipyretics
 - • 抗炎药anti-inflammatory drugs
 - • 利尿剂diuretics
 - • 充分休息adequate rest
 - • 良好护理good nursing care

预防与控制

Prevention and Control

- 新引进的马应隔离 3 至 4 周
- Newly introduced horses should be isolated for 3 to 4 weeks.
- 隔离急性感染马，以防止通过分泌物和排泄物传播。
- Acutely infected horses should be isolated to prevent transmission in secretions and excretions.
- 性传播可以通过良好的管理和疫苗接种来控制。
- Venereal transmission can be controlled by good management and vaccination.
- 为了保护怀母马，应该其他马匹分开并小群饲养
- To protect pregnant mares, they should be separated from other horses and maintained in small groups
- 种马携带者与未感染应实施物理隔离
- Carrier stallions should be physically separated from uninfected horses
- 筛查用于繁殖的种马或精液
- screen stallion or semen intended for breeding

诊断

Diagnosis

- 临床诊断：非特征性临床症状
- **Clinical Diagnosis: non-characteristic signs**
- 实验室检测
- --病原鉴定
- --血清型试验
- **Laboratory testing :**
- *--Identification of the agent*
- *--Serological tests*

鉴别诊断

Differential diagnosis

- 马病毒性动脉炎很难与呼吸道和非呼吸道马病的迹象相区分：马流感、马传染性贫血和非洲马瘟，以及盖塔病毒、亨德拉病毒、马腺病毒和马疱疹病毒 1 和 4 的感染。马病毒性动脉炎也类似于出血性紫癜 和其他链球菌感染。
- Infection with equine arteritis virus is difficult to differentiate from respiratory and non-respiratory equine diseases: equine influenza, equine infectious anemia and African horse sickness, as well as infections with Getah virus, Hendra virus, equine adenoviruses, and equine herpesviruses 1 and 4. Infection with equine arteritis virus also resembles purpura hemorrhagica and other streptococcal infections.

诊断

Diagnosis

- 临床诊断
- 当临床症状包括发烧、抑郁、水肿、结膜炎、流鼻涕和流产时，应考虑马病毒性动脉炎。
- 很难与马的其他全身和呼吸系统疾病相鉴别。
- ***Clinical Diagnosis***
- EAV should be considered when the clinical signs include fever, depression, edema, conjunctivitis, nasal discharges and abortions.
- This disease is difficult to differentiate from other systemic and respiratory illnesses of horses.

病毒分离

virus isolation and identification

- 细胞：兔、马和猴肾细胞或细胞系，常用 RK-13（兔肾）细胞
- 病毒鉴定：血清中和试验、RT-PCR、免疫荧光或免疫组织化学
- 病毒鉴定：RT-PCR 试验、中和试验、间接免疫荧光试验、免疫组织化学
- Cells：rabbit, equine and monkey kidney cells or cell lines. RK-13 (rabbit kidney) cells are the system of choice.
- Identification of Virus: *Confirmed by RT-PCR assay, neutralisation test, indirect immunofluorescence techniques, immuno-histochemistry.*

样品采集

Collection of samples for virus isolation or RT-PCR

- 新近感染动物：鼻腔分泌物、抗凝血和精液
- 种马携带者：精液；但在呼吸道分泌物、血液或尿液中未发现
- 疑似流产病例:胎盘组织和体液以及胎儿肺、肝、淋巴网状组织（尤其是胸腺）以及腹膜或胸膜液。绒毛尿囊膜和胎肺是病毒恢复的首选组织。
- 死亡的幼驹或老马：组织标本，尤其是胸腹腔及相关器官的淋巴腺
- Recently infected animals: nasal secretions, unclotted blood and semen
- Carrier stallions: semen, but not in the respiratory secretions, blood or urine.
- Suspect cases of abortion: placental tissues and fluids and from fetal lung, liver, lymphoreticular tissues (especially thymus), and peritoneal or pleural fluid. Chorioallantoic membrane and fetal lung are the tissues of choice for recovery of virus.
- Dead foals or older horses: tissue specimens, especially the lymphatic glands in the thoracic and abdominal cavities and related organs

样品采集：病毒分离、RT-PCR samples for virus isolation or RT-PCR

- 细胞：兔、马和猴肾细胞或细胞系，常用 RK-13（兔肾）细胞
- 病毒鉴定：血清中和试验、RT-PCR、免疫荧光或免疫组织化学
- Cells：rabbit, equine and monkey kidney cells or cell lines. RK-13 (rabbit kidney) cells are the system of choice.
- Identification of Virus: serum neutralization, RT-PCR, immunofluorescence or immuno-histochemistry.

样品采集：血清学试验

Samples: Serological tests

- 血清（2ml）：来源于促凝血（7-8ml）
- serum (2ml): from clotted blood samples 7-8ml
- 未免疫的马：单份血清
- For vaccinated horses: single serum sample
- 对免疫的马（国际调运）：间隔14天采集双份血清
- For non-vaccinated horses (international movement): paired samples at 14 days interval
- 急性病例（国内确诊）：间隔2-4周收集双份样品（急性期和恢复期血清）
- acute case (national confirmation): Paired samples (acute and convalescent serum) collected at an interval of 2-4 weeks

血清学试验

Serological tests

- 血清中和试验Serum neutralisation (VN)
- 补体结合试验Complement fixation (CF)
- 直接荧光抗体试验indirect fluorescent antibody (IFA)
- 琼脂凝胶免疫扩散试验agar gel immunodiffusion (AGID)
- 酶联免疫吸附试验enzyme-linked immunosorbent assay (ELISA)
- 荧光微球免疫试验fluorescent microsphere immunoassay (MIA)

血清中和试验

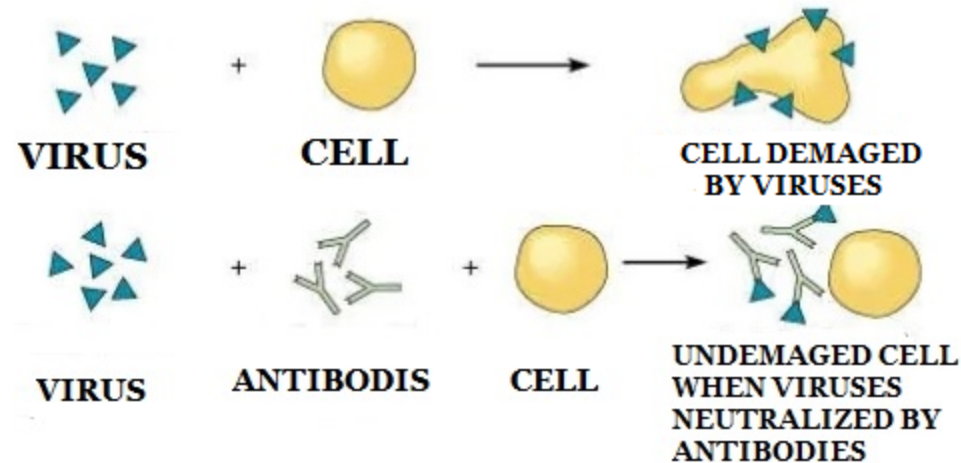
Serum neutralization [SN] test

- 血清中和试验 (SN) = 病毒中和试验 (VN)
- Serum neutralization [SN] = virus neutralisation (VN)
- 高度敏感性 : highly sensitive
- 高度特异性 : highly specific
- 用途 : 测定感染后或接种疫苗后中和抗体滴度
- Usage: Measure the titer of neutralizing antibodies post infection or after vaccination
-
- 金标准试验 : Gold standard test
- 最经典的试验 : Most classical test
- 确证试验 : Confirmation test
- 世界动物卫生组织 (OIE) 推荐: Recommended by World Organisation for Animal Health (OIE).

中和试验原理

Principle in Serum neutralization

NEUTRALIZATION



- 如果血清中存在抗体，就会与病毒结合，阻止病毒侵入细胞并阻断感染。细胞病变效应（CPE，感染引起的细胞变化）只有在细胞被感染时才会出现。
- If antibodies are present in the serum, they bind to the virus and prevent invasion and block infection of the cells. Cytopathic effects (CPE, changes in the cells from infection) are only seen if the cells are infected.

1.试验器材

1. Material

1.1液氮罐或-80冰箱

1.1 Liquid nitrogen tank or -80 refrigerator

1.2 CO₂培养箱

1.2 CO₂ incubator

1.3台式离心机

1.3 Tabletop centrifuge

1.4水浴锅

1.4. Water bath

1.5 倒置显微镜

1.5. Inverted microscope

1.6细胞瓶 (25 cm²)

1.6 Flask: tissue culture flasks (25 cm²)

1.7细胞板:96孔平底微量滴定板, 组织培养级

1.7Plate: flat-bottom 96-well microtiter assay plate, tissue culture grade

1.8微量移液器: 单道[200μL、1000-μL], 12道[200μL]

1.8 Micropipettors: 200 μL and 1000 μL single channel; 200-μL x 12-channel

1.9 种用细胞 (MCS): RK-13 (ATCC-CCL37), 购自美国典型菌种保藏中心 (ATCC)

1.9 Master cell stock (MCS): RK-13 (ATCC-CCL37), American Type Culture Collection (ATCC)

1.10病毒: 马动脉炎种毒CVL-Bucyrus株

1.10. Virus: equine arteritis virus, CVL-Bucyrus strain

1.11 最低需要基础培养基 (MEM)

1.11. Minimum Essential Medium (MEM)

1.12胎牛血清 (FCS)

1.12 Fetal calf serum [FCS]

1.13 稀释液: 无胎牛血清的MEM

1.13 Diluent: FCS-free MEM

1.14细胞维持液:含2%FCS的MEM+1% 100X抗生素储存液

1.14 Culture medium: MEM containing 10% FCS and 1% 100X antibiotics stock

1.15细胞培养液:含10%FCS的MEM+1% 100X抗生素储存液

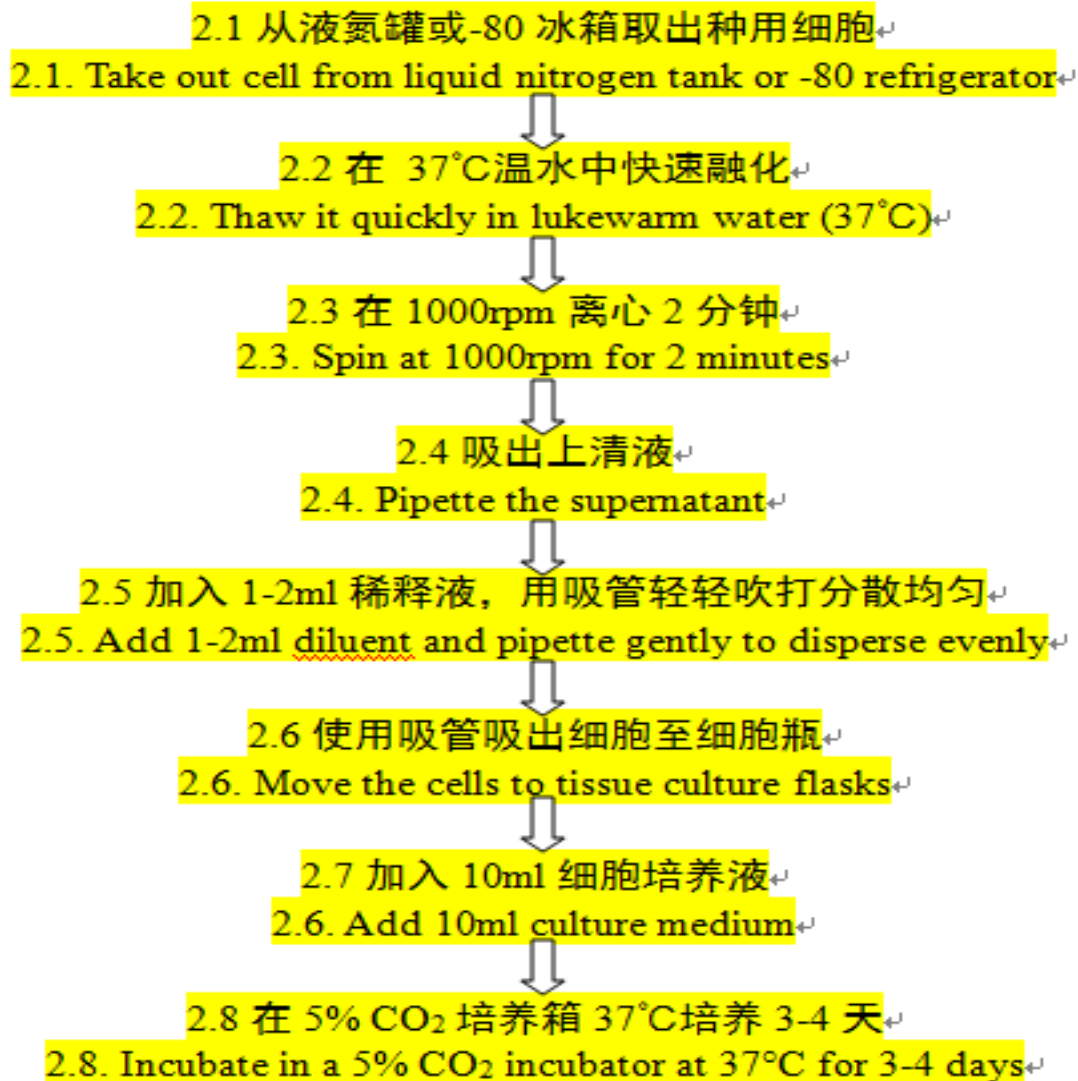
1.15 Maintenance medium: MEM containing 2% FCS and 1% 100X antibiotics stock

1.16抗生素储存液 (100X): 青霉素溶液 (100 00μg)、链霉素溶液 (100 00μg)

1.16 Antibiotics stock (100X): penicillin solution 100 00μg and streptomycin solution 100 00μg

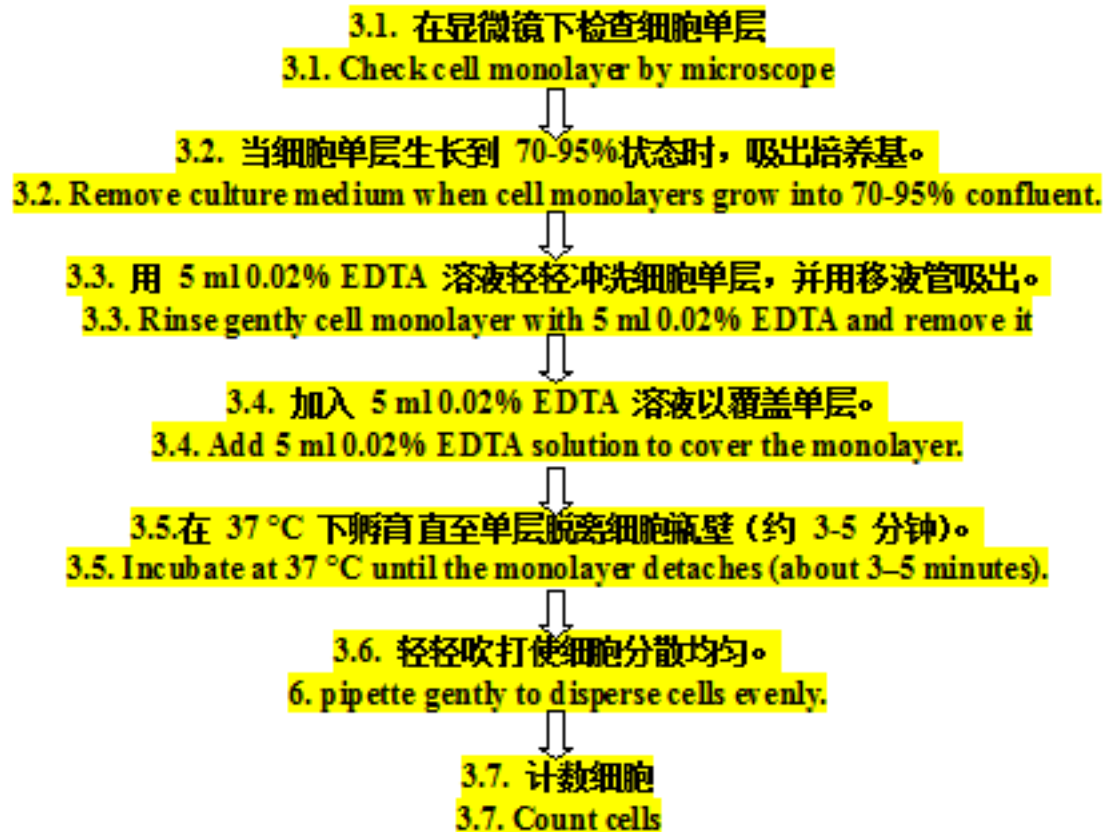
2.细胞准备

2. Cell preparation



3. 细胞消化

3.Cell digestion

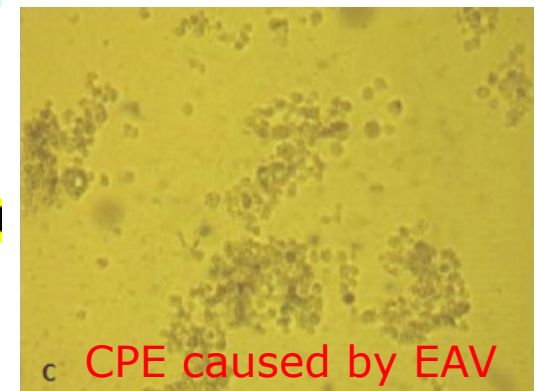
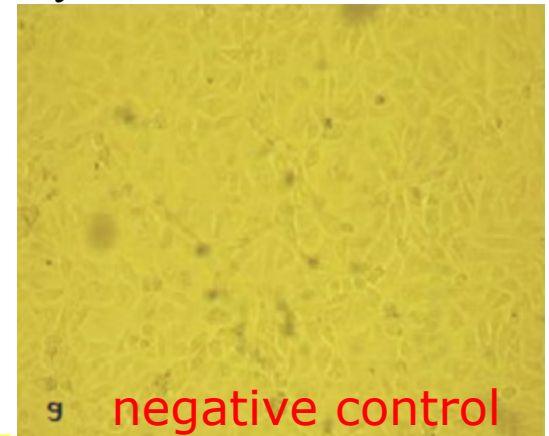


4.种毒制备

4. Seed virus preparation



马动脉炎病毒细胞病变 (CPE):
细胞溶解、死亡和脱落
Characteristic CPE in EAV infection:
cellular lysis, death and detachment of cells.



5. 病毒滴度测定

5. Determination of virus titer

5.1 在 96 孔微量细胞板上每孔加 25 μ L 稀释液

5.1. Add 25 μ L of diluent in all wells of microtiter plate;

5.2 将冻存的种毒作 10^{-1} 、 10^{-2} ... 10^{-8} 系列稀释

5.2. Make 10-fold serial dilution of the seed virus from 10^{-1} , 10^{-2} ,... 10^{-8}

5.3 每一稀释度接种 8 孔，每孔 25 μ L

5.3. Add 25 μ L of diluted virus in 8 wells for each dilution, i.e. eight 25 μ L replicates per dilution

5.4 加 100 μ L 的 RK-13 细胞悬液 (2.5×10^5 /mL) 于各孔中

5.4. Add 100 μ L of RK-13 cell suspension (2.5×10^5 /mL) in each well

5.5 在 5% CO_2 加湿的培养箱 37 $^\circ\text{C}$ 培养 3-4 天

5.5. Incubate the plate for 3-4 days at 37 $^\circ\text{C}$ in a humid atmosphere of 5% CO_2 in air.

5.6 每天观察 CPE

5.6. Observe daily all wells by microscopy for the CPE appearance

5.7 记录结果，按 Spearman-Kärber 法计算病毒滴度 TCID_{50}

5.7. Record results and calculate virus titer TCID_{50} by to Spearman-Kärber method.

应用 Spearman-Kärber法计算病毒滴度

Calculate virus titer by Spearman-Kärber method

应用 Spearman-Kärber 法计算病毒滴度

Log10 病毒稀释度	出现 CPE 孔数	总孔数	备注
-1	10	10	
-2	10	10	
-3	10	10	
-4	10	10	
-5	10	10	X=5
-6	6	10	
-7	1	10	10+6+1=17
-8	0	10	

Spearman-Kärber 公式

病毒滴度 = $(X - d/2 + [d \cdot S])$, 其中

--X = Log10 全部孔出现 CPE 的稀释度 CPE (=5)

--d = Log10 稀释倍数 (1)

--S = 从出现 CPE 的最高稀释度开始累计各稀释度出现 CPE 的孔比例总和 $([10+6+1]/10)$

Log10 50% 终点滴度 = $- [5 - \frac{1}{2} + 1 (17/10)] = -6.2$

病毒滴度 = $10^{6.2}$ TCID₅₀/mL

Calculation of virus titer using the Spearman-Kärber method

Log10 virus dilution	Wells showing CPE	Total of wells	Note
-1	10	10	
-2	10	10	
-3	10	10	
-4	10	10	
-5	10	10	X=5
-6	6	10	
-7	1	10	10+6+1=17
-8	0	10	

Spearman-Kärber formula

Virus titer = $(X - d/2 + [d \cdot S])$, where

--X = Log10 of dilution with all wells showing CPE (=5)

--d = Log10 of dilution factor (1)

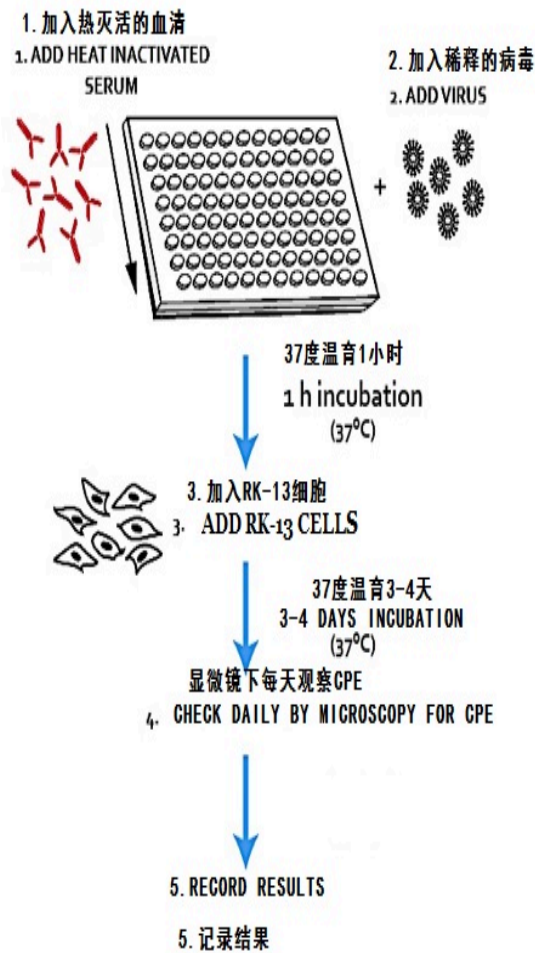
--S = Sum of proportions of wells showing CPE for all dilutions tested $([10+6+1]$

Log10 of 50% endpoint dilution = $- [5 - \frac{1}{2} + 1 (17/10)] = -6.2$

Titre of the virus = $10^{6.2}$ TCID₅₀/mL

6. 中和试验程序

6.serum neutralisation (SN) test



- 6.1 待检血清和对照血清在56°C水浴中灭活30分钟。
- 6.1. Inactivate test sera and control sera for 30 minutes in a water bath at 56°C.
- 6.2 在微量滴定板上，每孔加25μL无FCS的MEM。
- 6.2. Add 25 μ L of FCS-free MEM to all wells of the microtiter plates.
- 6.3 血清做倍比稀释，即混合后取25 μ L，加入下一孔，充分混合，同法依次稀释，直至最后一孔。每板可测定6个血清样。
- 6.3. Make doubling dilutions of each serum by sequential mixing and transfer of 25 μ L to each subsequent row of wells. Six sera can be assayed in each plate.
- 6.4 每孔加25 μ L稀释的病毒（100 TCID₅₀/孔）。
- 6.4. Add 25 μ L of diluted virus to each well (100 TCID₅₀/well).
- 6.5 滴定板在含5% CO₂的培养箱中37°C孵育1小时。
- 6.5. Incubate the microtiter plates for 1 hour at 37°C in 5% CO₂ atmosphere.
- 6.6 每孔加100μL RK-13细胞悬液（用细胞培养液配成 5×10^5 个细胞/mL）。
- 6.6. Add 100 μ L of RK-13 cell suspension (5×10^5 cells/mL) to each well.
- 6.7 滴定板在含5% CO₂的培养箱中37°C孵育3~4天。
- 6.7. Incubate the plates for 3-4 days at 37°C in an atmosphere of 5% CO₂ in air.
- 6.8 显微镜下检查CPE，记录实验结果。
- 6.8. Examine the plates by microscopy for CPE and record the results on a worksheet.

注意：出于安全原因，所有试验必在二级生物安全柜中进行，以防止细胞污染和病毒扩散。

Note: for safety reasons, all test procedures must be performed in a class-II biosafety cabinet to prevent the contamination of cells and virus spread.

检查所有试验对照

Check all controls

6.10 检查所有试验对照正常且种毒滴度为30–300 TCID₅₀。↵

6.10. The validity of the test is confirmed by establishing that all controls are normal and the working dilution of seed virus contained 30–300 TCID₅₀ virus.↵

↵

6.10.1 细胞对照（无病毒）：细胞单层完整，无CPE↵

6.10.1. cell control (no virus): intact cell monolayers without CPE↵

↵

6.10.2 血清毒性对照：细胞单层完整，无CPE ↵

6.10.2. serum cytotoxicity control: intact cell monolayers without CPE↵

↵

6.10.3 阳性血清对照：细胞单层完整，无CPE↵

6.10.3. positive serum control: intact cell monolayers without CPE↵

↵

6.10.4 阴性血清对照：细胞单层破坏，出现CPE↵

6.10.4. negative serum control: destroyed cell monolayers with CPE↵

↵

6.10.5 病毒对照（病毒回归滴定）：每孔加入的病毒量在30–300 TCID₅₀之间↵

6.10.5. Virus control (virus back titration): Make sure that actual amount of virus added to each well is between 30–300 TCID₅₀.↵

↵

7.议定书要求

7. Requirements in the Protocol

- 7.1 未免疫的马，血清中和试验，血清1:4稀释，结果为阴性；或
- 7.1. For vaccinated horses, serum neutralisation test at a serum dilution of 1:4 with negative result ; OR
-
- 7.2 免疫的马，间隔14天采集双份血清，做血清中和试验，抗体滴度稳定不变或降低，并采集抗凝血进行RT-PCR试验或病毒分离结果为阴性。
- 7.2. For non-vaccinated horses, serum neutralization tests of paired samples at 14 days interval, the second result which demonstrated stable or declining antibody titres, and the animals shall be subject to RT-PCR test or virus isolation test with negative results, using unclotted blood samples.

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THANKS