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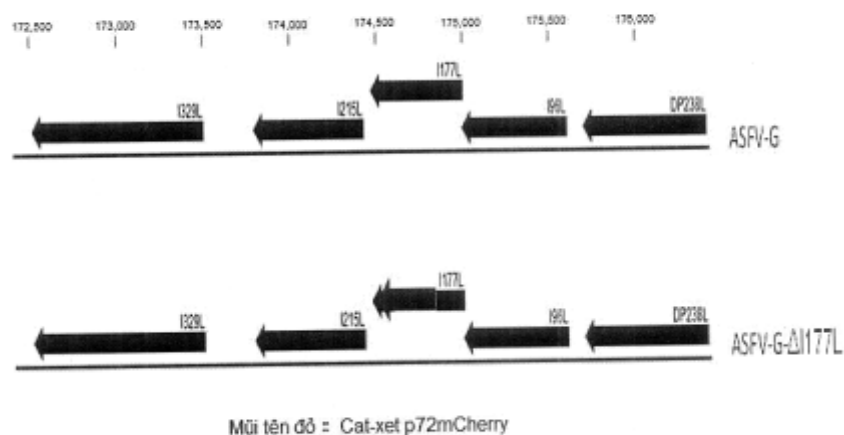
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(57) Sáng chế đề cập đến các chi tiết về việc chế tạo vaccin sống giảm độc lực của Virut gây bệnh tả lợn Châu Phi (ASFV) tái tổ hợp để phòng ngừa bệnh ASF do các chủng ASFV khác nhau gây ra, chẳng hạn như chủng phân lập Georgia 2007 có độc lực cao (“ASFV-G”). Vaccin mẫu bao gồm virut đã được sửa đổi ASFV-GΔI177L, ASFV-G tái tổ hợp được sửa đổi bằng cách xóa một phần của I177L ORF làm cho gen I177L không hoạt động.



HÌNH 1

Lĩnh vực kỹ thuật được đề cập

Sáng chế đề cập đến thông tin chi tiết về việc chế tạo vacxin sống giảm độc lực của Virut gây bệnh tả lợn Châu Phi (ASFV) tái tổ hợp để phòng ngừa bệnh ASF do các chủng ASFV khác nhau gây ra, chẳng hạn như chủng phân lập Georgia 2007 có độc lực cao (“ASFV-G”). Vacxin mẫu bao gồm virut đã được sửa đổi ASFV-GΔI177L, ASFV-G tái tổ hợp được sửa đổi bằng cách xóa một phần của I177L ORF làm cho gen I177L không hoạt động.

Tình trạng kỹ thuật của sáng chế

Bệnh tả lợn châu Phi (ASF) là một bệnh do virut truyền nhiễm ở lợn. Tác nhân gây bệnh, virut ASF (ASFV), là một loại virut có vỏ bọc lớn chứa bộ gen ADN sợi đôi gồm khoảng 190 cặp kilobase. ASFV chia sẻ các khía cạnh của cấu trúc bộ gen và chiến lược sao chép với các virut ADN sợi đôi lớn khác, bao gồm Poxviridae, Iridoviridae và Phycodnaviridae (Costard và cộng sự, Phil. Trans. Royal Soc. B, (2009) 364: 2683-96). Nhiễm ASFV ở lợn nhà thường gây tử vong và có điểm khác biệt ở sốt, xuất huyết, mất điều hòa và suy nhược nghiêm trọng. Tuy nhiên, quá trình lây nhiễm khác nhau, từ gây tử vong cao đến cận lâm sàng, tùy thuộc vào đặc điểm vật chủ và chủng virut cụ thể (Tulman và cộng sự, Curr. Top. Microbial. Immunol. (2009) 328: 43-87).

Hiện nay, căn bệnh này lưu hành ở hơn 20 quốc gia châu Phi cận Sahara. Ở châu Âu, ASF vẫn còn lưu hành trên đảo Sardinia (Ý) và các đợt bùng phát mới đã được công bố ở khu vực Kavkaz từ năm 2007, ảnh hưởng đến Gruzia, Armenia, Azerbaijan và Nga. Các đợt bùng phát gần đây đã được báo cáo ở Ukraine, Belarus, Lithuania, Latvia và Ba Lan, ảnh hưởng đến cả các trang trại lợn rừng và lợn nhà. Trong năm 2018-2019, ASF đã lan sang Trung Quốc, Mông Cổ, Việt Nam, Campuchia và Triều Tiên, ở cả các trang trại nuôi lợn rừng và lợn nhà. Vào năm 2019, ASF cũng đã lây lan sang các quần thể lợn rừng ở Bỉ, nơi ASF hiện chỉ ảnh hưởng đến một khu vực ngăn chặn nhỏ trong nước. Các đợt bùng phát ASF gần đây có nguy cơ lây lan sang các nước láng giềng. Virut gây bệnh dịch ASFV Georgia 2007/1 bố mẹ, là một chủng phân lập có độc lực cao thuộc kiểu gen II (Chapman và cộng sự, Emerging Infect. Dis. (2011) 17: 599-605), và là nguyên nhân gây ra tất cả các vụ bùng phát hiện nay ở Châu Á và Châu Âu, với các virut bùng phát có độ giống từ 90% trở lên với chủng gốc.

Hiện tại, không có vacxin thương mại cho ASF và dịch bệnh được kiểm soát bởi kiểm dịch động vật và giết mổ. Nỗ lực tiêm phòng cho động vật bằng cách sử dụng

chất chiết xuất từ tế bào bị nhiễm bệnh, dịch nổi của bạch cầu máu ngoại vi lợn bị nhiễm bệnh, virion tinh khiết và bất hoạt, đại thực bào cố định glutaraldehyt bị nhiễm bệnh hoặc đại thực bào phế nang bị nhiễm chất tẩy rửa không tạo được miễn dịch bảo vệ (Coggins, L., *Prag. Med. Viral.* (1974) 18: 48-63; Forman và cộng sự, *Arch. Viral.*, (1982) 74: 91-100; Kihm và cộng sự, (1987) Trong: *African Swine Fever*, Becker, Y. (ed), Martinus Nijhoff, Boston, trang 127-44; Mebus, C. A., *Adv. Virut Res.*, (1988) 35: 251-69). Miễn dịch bảo vệ đồng loại không phát triển ở lợn sống sót sau khi nhiễm virut. Những con lợn sống sót sau đợt nhiễm trùng cấp tính với các biến thể độc lực trung bình hoặc giảm độc lực của ASFV phát triển khả năng kháng lâu dài đối với virut đồng loại, nhưng hiếm khi chống lại sự thách thức của virut (Hamdy và Dardiri, *Am. J. Vet. Res.* (1984) 45: 711-14; Ruiz-Gonzalvo và cộng sự, (1981) Trong: *FAO/CEC Expert Consultation in ASF Research*, Wilkinson, P. J. (ed), Rome, trang 206-16). Trong tài liệu này, chủ đơn báo cáo sự phát triển của vaccin tái tổ hợp trong đó một phần của gen I177L đã bị xóa khỏi bộ gen ASFV-G. Việc tiêm vaccin cho lợn bằng loại vi-rút này đã bảo vệ lợn khỏi phát triển ASF. Bởi vì hiện tại không có vaccin ASFV, sự phát triển của bất kỳ loại vaccin nào có thể tạo ra sự bảo vệ chống lại sự biểu hiện gây chết người của căn bệnh này đang rất được quan tâm.

Bản chất kỹ thuật của sáng chế

Sáng chế đề cập đến virut đã biến đổi gen, trong đó virut bao gồm bộ gen của virut giống ít nhất 99% với SEQ ID NO: 2. Theo một phương án cụ thể, bộ gen của virut bao gồm SEQ ID NO: 2.

Sáng chế còn đề cập đến chế phẩm vaccin chống lại virut gây bệnh Dịch tả lợn Châu Phi (ASFV), bao gồm virut đã được biến đổi gen bao gồm bộ gen virut giống ít nhất 99% với SEQ ID NO: 2. Theo một số phương án, ASFV là chủng phân lập ASFV- Georgia 2007.

Sáng chế còn đề cập đến phương pháp bảo vệ lợn chống lại ASFV, bao gồm việc sử dụng vaccin sống giảm độc lực cho lợn chứa virut biến đổi gen, trong đó virut này bao gồm bộ gen virut giống ít nhất 99% với SEQ ID NO: 2 với một lượng hiệu quả để bảo vệ lợn khỏi bệnh ASFV lâm sàng. Theo một số phương án, ASFV là ASFV-G. Theo các phương án cụ thể, lượng hiệu quả để bảo vệ lợn khỏi bệnh ASFV lâm sàng là vaccin chứa 10^2 - 10^6 HAD₅₀ của virut biến đổi gen.

Phương án bổ sung được đề cập là virut đột biến ASFV tái tổ hợp, bao gồm đột biến tổng hợp trong khung đọc mở I177L hoặc trong yếu tố điều hòa kiểm soát sự biểu hiện của protein I177L, dẫn đến gen I177L bộ gen không có chức năng. Theo các phương án cụ thể, đột biến tổng hợp là đột biến mất đoạn dẫn đến mất một hoặc nhiều nucleotit giữa các vị trí 174471 và 175004 của SEQ ID NO: 1. Theo các phương án khác, đột biến tổng hợp là đột biến lệch khung, đột biến chèn, đột biến vô nghĩa của

một hoặc nhiều nucleotit giữa vị trí 174471 và 175004 của SEQ ID NO: 1. Theo một số phương án, ASFV đột biến là chủng phân lập ASFV-Georgia. Theo các phương án cụ thể, ASFV đột biến bao gồm bộ gen giống ít nhất 95% hoặc giống ít nhất 99% với SEQ ID NO: 2.

Được cung cấp thêm ở đây là chế phẩm vaccin chống lại ASFV-G, chứa virut đột biến ASFV tái tổ hợp, chứa đột biến tổng hợp trong khung đọc mở I177L hoặc trong yếu tố điều hòa kiểm soát sự biểu hiện của protein I177L, dẫn đến gen I177L bộ gen không có chức năng.

Sáng chế còn đề cập đến phương pháp bảo vệ lợn chống lại ASFV, bao gồm việc sử dụng vaccin sống giảm độc lực cho lợn chứa virut đột biến ASFV tái tổ hợp, bao gồm đột biến tổng hợp trong khung đọc mở I177L hoặc trong yếu tố điều chỉnh kiểm soát sự biểu hiện của I177L protein, tạo ra một gen I177L bộ gen không có chức năng với số lượng có hiệu quả để bảo vệ lợn nói trên khỏi bệnh ASFV lâm sàng. Theo các phương án cụ thể, ASFV là ASFV-G. Theo một số phương án, lượng có hiệu quả để bảo vệ lợn khỏi bệnh ASFV lâm sàng là vaccin chứa 10^2 - 10^6 HAD₅₀ thuộc loại biến đổi gen.

KẾT HỢP THÔNG QUA THAM CHIẾU

Tất cả các ấn phẩm, bằng sáng chế và đơn đăng ký sáng chế được đề cập trong bản mô tả này được đưa vào đây bằng cách tham chiếu ở mức độ tương tự như thể mỗi công bố, bằng sáng chế hoặc đơn đăng ký sáng chế được chỉ định cụ thể và riêng lẻ để kết hợp thông qua tham chiếu.

Mô tả vắn tắt các hình vẽ

Đơn đăng ký bảo hộ sáng chế có ít nhất một bản vẽ màu. Văn phòng sẽ cung cấp các bản sao của bằng sáng chế hoặc đơn đăng ký sáng chế này với (các) bản vẽ màu theo yêu cầu và thanh toán lệ phí cần thiết.

Các tính năng mới của sáng chế được đưa ra với sự đặc biệt trong các yêu cầu bảo hộ. Các tính năng và ưu điểm của sáng chế được đề cập đến trong phần mô tả chi tiết sau đây và các bản vẽ kèm theo:

Hình 1 cung cấp hình ảnh minh họa của cat-xet được sử dụng để tạo virut đột biến tái tổ hợp ASFV-G Δ I177L.

Hình 2 biểu diễn đồ thị về các đặc điểm tăng trưởng trong ống nghiệm của ASFV-G- Δ I177L và ASFV-G gốc. Nuôi cấy tế bào đại thực bào lợn sơ cấp đã bị nhiễm (MOI = 0,01) với mỗi loại virut và sản lượng virut được chuẩn độ tại các thời điểm chỉ định sau khi nhiễm bệnh. Dữ liệu đại diện cho các phương tiện từ ba thí nghiệm độc lập. Độ nhạy phát hiện virut: $\geq 1.8 \log_{10}$ HAD₅₀/ml.

Hình 3 biểu diễn đồ thị của các hiệu giá trạng thái virus xâm nhập vào dòng máu (viremia) được phát hiện ở lợn được cấy IM với 10^2 HAD₅₀ của ASFV-G-ΔI177L hoặc 10^2 HAD₅₀ của ASFV-G. Mỗi đường cong thể hiện giá trị của từng con vật trong mỗi nhóm. Độ nhạy phát hiện virus: $\geq \log_{10} 1.8 \log_{10} \text{TCID}_{50}/\text{ml}$.

Hình 4 biểu diễn đồ thị động học của các giá trị nhiệt độ cơ thể ở lợn được cấy IM bằng 10^2 , 10^4 hoặc 10^6 HAD₅₀ của ASFV-G-ΔI177L (ký hiệu đầy đủ), mô hình được cấy (dự phòng, được hiển thị bằng màu đỏ) hoặc 10^2 HAD₅₀ của ASFV-G (biểu tượng trống) (bảng bên trái) và sau thử thách với 10^2 HAD₅₀ của ASFV-G (bảng bên phải). Mỗi đường cong thể hiện giá trị của từng con vật trong mỗi nhóm.

Hình 5 biểu diễn đồ thị của các hiệu giá viremia được phát hiện ở lợn được cấy IM với 10^2 , 10^4 , hoặc 10^6 HAD₅₀ của ASFV-G-ΔI177L hoặc 10^2 HAD₅₀ của ASFV-G. Viremia sau thử thách với 10^2 HAD₅₀ của ASFV-G. Mỗi đường cong thể hiện giá trị của từng con vật trong mỗi nhóm. Độ nhạy phát hiện virus: $\geq \log_{10} 1.8 \text{TCID}_{50}/\text{ml}$.

Hình 6 cung cấp biểu diễn đồ họa của kháng thể kháng ASFV (trung gian IgM hiển thị trong bảng ở cột bên trái và IgG qua trung gian hiển thị trong bảng ở cột bên phải) hiệu giá phát hiện bằng ELISA ở lợn được cấy IM bằng 10^2 , 10^4 , or 10^6 HAD₅₀ của ASFV-G-ΔI177L. Đáp ứng kháng thể qua trung gian IgM Mỗi đường cong thể hiện giá trị của từng con vật trong mỗi nhóm..

Mô tả chi tiết sáng chế

Virus gây bệnh dịch tả lợn Châu Phi (ASFV) là tác nhân gây bệnh của loại bệnh do virus truyền nhiễm và thường gây chết ở lợn nuôi gây hậu quả kinh tế đáng kể cho ngành chăn nuôi lợn. Việc kiểm soát bệnh Dịch tả lợn Châu Phi (ASF) đã bị cản trở do không có vaccin. Các vaccin thử nghiệm đã được báo cáo trước đây là có nguồn gốc từ ASFV sống giảm độc lực tự nhiên, thích nghi với nuôi cấy tế bào hoặc biến đổi gen. Tuy nhiên, không có loại vaccin nào trong số này được phát triển để sử dụng cho mục đích thương mại. Ở đây chủ đơn báo cáo phát hiện rằng việc xóa một gen chưa được xác định trước đây, I177L, khỏi chủng phân lập ASFV độc lực cao của Georgia (ASFV-G) tạo ra sự suy yếu hoàn toàn ở lợn. Động vật được tiêm virus thiếu gen I177L chức năng - chẳng hạn như đột biến ASFV-G-ΔI177L cụ thể được mô tả ở đây - được tiêm bắp (IM) vẫn bình thường về mặt lâm sàng trong thời gian quan sát 28 ngày. Điều quan trọng là, động vật bị nhiễm ASFV-G-ΔI177L đã được bảo vệ khi thử thách với chủng gốc độc lực ASFV-G.

Các phương án tốt hơn theo sáng chế được chỉ ra và mô tả ở đây. Đối với những người hiểu biết trong lĩnh vực kỹ thuật này sẽ hiển nhiên rằng các phương án như vậy chỉ được cung cấp bằng cách nêu ví dụ. Nhiều biến thể, thay đổi và thay thế sẽ xảy ra đối với những người có hiểu biết trong lĩnh vực kỹ thuật này không tách rời khỏi sáng

chế. Các phương án thay thế khác nhau cho các phương án của sáng chế được mô tả ở đây có thể được sử dụng trong việc thực hành sáng chế. Mục đích là các yêu cầu bảo hộ được bao gồm tại đây xác định phạm vi của sáng chế và do đó các phương pháp và cấu trúc trong phạm vi của các yêu cầu bảo hộ này và các yêu cầu bảo hộ tương đương của chúng được đề cập đến.

Các thuật ngữ khoa học và kỹ thuật được sử dụng ở đây có nghĩa thường được hiểu bởi người có hiểu biết trung bình trong lĩnh vực kỹ thuật mà sáng chế đề cập đến, trừ khi được định nghĩa khác. Ở đây đưa ra tham chiếu đến các tài liệu và phương pháp luận khác nhau được biết đến với những người có hiểu biết trong lĩnh vực kỹ thuật. Các công trình tham khảo tiêu chuẩn đưa ra các nguyên tắc chung của công nghệ ADN tái tổ hợp bao gồm Sambrook và cộng sự, "Molecular Cloning: A Laboratory Manual", ấn bản lần 2, Cold Spring Harbor Laboratory Press, Plainview, N.Y., 1989; Kaufman và cộng sự, tái bản, "Handbook of Molecular and Cellular Methods in Biology and Medicine", Nhà xuất bản CRC, Boca Raton, 1995; và McPherson, "Directed Mutagenesis: A Practical Approach", Nhà xuất bản IRL, Oxford, 1991.

Bất kỳ vật liệu và/hoặc phương pháp thích hợp nào mà những người có hiểu biết trong lĩnh vực kỹ thuật đã biết đều có thể được sử dụng để thực hiện sáng chế tức thì. Các tài liệu và/hoặc phương pháp thực hành sáng chế tức thì được mô tả. Các vật liệu, thuốc thử và những thứ tương tự được đề cập đến trong phần mô tả và ví dụ sau đây có thể lấy được từ các nguồn thương mại, trừ khi có ghi chú khác. Sáng chế này hướng dẫn các phương pháp và mô tả các công cụ để tạo ra các chủng ASFV đã biến đổi gen.

Như được sử dụng trong đặc tả và tuyên bố, việc sử dụng số ít "cái", "một" và "cái này" bao gồm các tham chiếu số nhiều trừ khi ngữ cảnh quy định rõ ràng theo cách khác.

Các thuật ngữ được phân lập, tinh khiết hoặc tinh khiết sinh học như được sử dụng ở đây, đề cập đến vật liệu về cơ bản hoặc không có các thành phần thường đi kèm với vật liệu được tham chiếu ở trạng thái ban đầu của nó.

Thuật ngữ "khoảng" được định nghĩa là cộng hoặc trừ mười phần trăm của một giá trị được đọc. Ví dụ: khoảng 1,0g có nghĩa là 0,9g đến 1,1g và tất cả các giá trị trong phạm vi đó, cho dù được nêu cụ thể hay không.

Thuật ngữ "axit nucleic về cơ bản bao gồm", và các biến thể ngữ pháp của chúng, có nghĩa là các axit nucleic khác với trình tự axit nucleic tham chiếu bằng 20 gốc axit nucleic trở xuống và cũng thực hiện chức năng của trình tự axit nucleic tham chiếu. Các biến thể như vậy bao gồm các trình tự ngắn hơn hoặc dài hơn trình tự axit

nucleic tham chiếu, có các gốc khác nhau ở các vị trí cụ thể hoặc sự kết hợp của chúng.

Thuật ngữ “chất bổ trợ” có nghĩa là một chất hoặc phương tiện không đặc biệt giúp tăng cường phản ứng miễn dịch đối với kháng nguyên. Chất bổ trợ có thể bao gồm hỗn dịch khoáng (phèn, nhôm hydroxit, hoặc phosphat) mà trên đó kháng nguyên được hấp thụ; hoặc nhũ tương nước trong dầu, trong đó dung dịch kháng nguyên được nhũ hóa trong dầu khoáng (ví dụ, chất bổ trợ không hoàn chỉnh của Freund), đôi khi có bao gồm cả vi khuẩn mycobacteria bị tiêu diệt (chất bổ trợ hoàn chỉnh của Freund) để tăng cường thêm tính kháng nguyên. Các oligonucleotit kích thích miễn dịch cũng có thể được sử dụng làm chất bổ trợ (ví dụ: xem các bằng sáng chế Hoa Kỳ số 6,194,388; 6,207,646; 6,214,806; 6,218,371; 6,239,116; 6,339,068; 6,406,705; và 6,429,199). Chất bổ trợ cũng bao gồm các phân tử sinh học, chẳng hạn như các phân tử kích thích vũ trụ.

Thuật ngữ “truyền”/“sự truyền” có nghĩa là bất kỳ phương pháp nào cung cấp cho đối tượng một chất, chẳng hạn như chất điều trị bằng bất kỳ con đường hiệu quả nào. Các đường dùng ví dụ bao gồm, nhưng không giới hạn ở, đường tiêm (chẳng hạn như đường tiêm dưới da, tiêm bắp, trong da, trong phúc mạc và tĩnh mạch), đường uống, đường dẫn truyền, đường dưới lưỡi, trực tràng, qua da, đường mũi, đường âm đạo và đường hít.

Thuật ngữ “trình tự mã hóa” và “vùng mã hóa” như được sử dụng ở đây đề cập đến trình tự nucleotit và trình tự axit nucleic, bao gồm cả ARN và ADN, mã hóa thông tin di truyền để tổng hợp ARN, protein hoặc bất kỳ phần nào của ARN hoặc chất đậm.

Thuật ngữ “lượng hữu hiệu” của chế phẩm được đề cập ở đây dùng để chỉ lượng chế phẩm có khả năng thực hiện chức năng cụ thể mà lượng hữu hiệu được thể hiện. Lượng chính xác cần thiết có thể khác nhau giữa các thành phần và từ chức năng này sang chức năng khác, tùy thuộc vào các biến được công nhận như các thành phần và quá trình liên quan. Tổng hiệu quả có thể được phân phối trong một hoặc nhiều ứng dụng. Do đó, không thể xác định một lượng chính xác, tuy nhiên, một “lượng hữu hiệu” thích hợp có thể được xác định bởi các nghệ nhân lành nghề thông qua thử nghiệm thường xuyên.

Các thuật ngữ “I177L”, “ASFV I177L” và “hệ gen học I177L” là các từ đồng nghĩa và dùng để chỉ gen được định nghĩa ở đây là SEQ ID NO: 3 hoặc bất kỳ phiên bản nào của SEQ ID NO: 3 với sự thay thế bazơ dẫn đến protein có một trình tự giống với SEQ ID NO: 4) Các thuật ngữ này, trong ngữ cảnh thích hợp, cũng có thể đề cập đến các phiên bản sửa đổi của các SEQ ID NO này, chẳng hạn như các phiên bản bao gồm xóa, chèn và các sửa đổi tái tổ hợp khác. Khung đọc mở ASFV-G I177L mã hóa

một protein 177 axit amin (SEQ ID NO: 4) và được định vị trên sợi ngược giữa các vị trí nucleotit 174471 và 175004 của SEQ ID NO: 1

Trong ngữ cảnh của sáng chế, thuật ngữ "bộ gen I177L không có chức năng" dùng để chỉ gen I177L đã được sửa đổi, nằm trong bộ gen của ASFV, trong đó việc sửa đổi như vậy của gen ASFV I177L không tạo ra sản phẩm gen ASFV I177L nào cả hoặc một sản phẩm của gen ASFV I177L không có chức năng sinh học so với gen ASFV I177L có chức năng không bị biến đổi. Những sửa đổi như vậy có thể bao gồm, nhưng không giới hạn ở việc xóa toàn bộ hoặc một phần trình tự mã hóa, làm gián đoạn khung đọc mở (ví dụ: bằng cách chèn đột biến dịch chuyển hoặc chèn một codon vô nghĩa), sửa đổi các yếu tố quy định ngược dòng hoặc xuôi dòng, và/hoặc bất kỳ phương pháp nào khác hiện đã được biết đến hoặc có thể hình dung được để bất hoạt hoặc loại bỏ biểu hiện chức năng của gen ASFV I177L đó.

Thuật ngữ "chủng ngừa" có nghĩa là đề chỉ một đối tượng được bảo vệ khỏi bệnh truyền nhiễm, chẳng hạn như bằng cách tiêm chủng.

Theo mục đích của sáng chế này, "nhận dạng trình tự" của hai trình tự nucleotit hoặc axit amin liên quan, được biểu thị bằng phần trăm, đề cập đến số vị trí trong hai trình tự được căn chỉnh tối ưu có gốc giống nhau (x100) chia cho số các vị trí được so sánh. Khoảng trống, tức là một vị trí trong một hướng thẳng hàng trong đó chất căn có mặt trong một dãy nhưng không nằm trong dãy khác được coi là vị trí có các chất căn không giống nhau. Sự căn chỉnh của hai chuỗi được thực hiện bởi thuật toán Needleman và Wunsch (Needleman và Wunsch, J Mol Biol, (1970) 48: 3, 443-53). Căn chỉnh trình tự có sự hỗ trợ của máy tính có thể được thực hiện thuận tiện bằng cách sử dụng chương trình phần mềm tiêu chuẩn như GAP, một phần của Gói Wisconsin Phiên bản 10.1 (Genetics Computer Group, Madison, Wisconsin, USA) bằng cách sử dụng ma trận tính điểm mặc định với hình phạt tạo khoảng cách là 50 và một hình phạt mở rộng khoảng cách là 3.

Cụm từ "phần trăm giống nhau cao" hoặc "phần trăm đặc điểm cao" và các biến thể ngữ pháp của chúng trong ngữ cảnh của hai polynucleotit hoặc polypeptit, đề cập đến hai hoặc nhiều trình tự hoặc trình tự con có ít nhất khoảng 80%, đồng nhất, ít nhất là khoảng 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% hoặc 100% nucleotit hoặc axit amin nhận dạng, khi được so sánh và căn chỉnh để có sự tương ứng tối đa, như được đo bằng thuật toán so sánh trình tự hoặc bằng cách kiểm tra trực quan. Theo một phương án được lấy làm ví dụ, các trình tự này có phần trăm giống nhau cao trên toàn bộ chiều dài của trình tự polynucleotit hoặc polypeptit.

Thuật ngữ "lợn" thường có thể đề cập đến bất kỳ thành viên nào của họ Suidae và bao gồm lợn thuần hóa, lợn rừng, lợn đực đã được thuần và lợn đực không bị thuần.

“Vaccin” ở đây được định nghĩa là tác nhân sinh học có khả năng cung cấp phản ứng bảo vệ ở động vật mà nó đã được truyền nhưng không có khả năng gây ra bệnh nghiêm trọng. Việc sử dụng vaccin dẫn đến khả năng miễn dịch khỏi bệnh. Do đó, vaccin kích thích sản xuất kháng thể hoặc miễn dịch tế bào chống lại mầm bệnh gây bệnh (ví dụ, ASFV). Miễn dịch ở đây được định nghĩa là việc tạo ra mức độ bảo vệ cao hơn đáng kể đối với khả năng gây chết và các triệu chứng lâm sàng sau khi tiêm phòng ở quần thể lợn, so với nhóm không được tiêm phòng. Đặc biệt, vaccin theo sáng chế bảo vệ hầu hết các động vật được tiêm phòng chống lại sự phát triển của các triệu chứng lâm sàng và khả năng gây chết của bệnh. Vaccin theo sáng chế thường là vaccin virus đột biến được biến đổi gen (tái tổ hợp).

Trong ngữ cảnh của sáng chế, thuật ngữ “không thiếu sự sao chép của nó” dùng để chỉ một ASFV tái tổ hợp không xuất hiện tự nhiên có thể sao chép trong ống nghiệm và/hoặc nghiên cứu trên động vật và/hoặc có khả năng tạo ra thể hệ virus mặc dù như vậy sự nhân lên và/hoặc sản xuất thể hệ con cháu của virus cũng có thể xảy ra ở mức độ giảm so với dòng bố mẹ không biến đổi. Do đó, có thể xảy ra trường hợp ASFV đó không bị thiếu hụt trong quá trình sao chép trong ống nghiệm, ví dụ: trong nuôi cấy tế bào, mặc dù trong cơ thể động vật có vú như vậy ASFV ít nhất bị suy giảm một phần trong quá trình sao chép của nó, ví dụ: dẫn đến sự nhân lên và/hoặc sản xuất thể hệ con cháu của virus dưới giới hạn phát hiện.

Như được sử dụng ở đây, thuật ngữ “liều lượng tối thiểu” hoặc “liều lượng hiệu quả tối thiểu” dùng để chỉ liều lượng chứng tỏ không có hoặc ít có độc tính đối với người nhận, nhưng vẫn tạo ra kết quả mong muốn (ví dụ: miễn dịch bảo vệ).

Virut/Vaccin

Được đề cập ở đây là virus ASFV-G Δ I177L đột biến mới (SEQ ID NO: 2), là kết quả của sự xóa tái tổ hợp một phần của gen I177L (SEQ ID NO: 3) của bộ gen ASFV-G của gen bố mẹ (SEQ ID NO: 1). Trình tự nucleotit bộ gen của một thể đột biến tái tổ hợp cụ thể ASFV-G Δ I177L (SEQ ID NO: 2) được mô tả ở đây và khác với trình tự nucleotit của bộ gen mã hóa ASFV-G của gen bố mẹ (SEQ ID NO: 1). Protein được mã hóa ASFV-G I177L gồm 177 axit amin (SEQ ID NO: 4) khác với protein I177L đột biến được dự đoán được mã hóa bởi trình tự nucleotit đột biến của ASFV-G Δ I177L. Protein I177L (SEQ ID NO: 6) từ ASFV-G Δ I177L được dự đoán là thiếu axit amin từ 112 đến 150 của protein I177L kiểu hoang dã. Vì cat-xet p72Mcherry được lắp vào vị trí này (xem phần Ví dụ), người ta không tin rằng vùng mã hóa còn lại sau khi chèn này được phiên mã, dẫn đến không có protein I177L chức năng nào được tạo ra trong quá trình lây nhiễm virus.

Chủng đột biến mẫu (ASFV-G Δ I177L (SEQ ID NO: 2)) là đại diện của giống vaccin tái tổ hợp trong đó gen ASFV I177L không có chức năng, bao gồm

nhưng không giới hạn, đột biến mất đoạn, đột biến vô nghĩa, đột biến chèn, đột biến lệch khung và các đột biến khác dẫn đến không biểu hiện protein I177L hoặc biểu hiện protein I177L không có chức năng. Các virus tái tổ hợp khác được hình dung bao gồm các đột biến trong các yếu tố điều hòa dẫn đến không biểu hiện hoặc không dịch mã của protein I177L.

Các sửa đổi nhằm mục đích ngăn cản sự biểu hiện chức năng của protein mục tiêu (ví dụ, I177L) hoặc giảm biểu hiện hoặc giảm hoạt tính của protein mục tiêu có thể liên quan đến các đột biến của ADN hoặc gen mã hóa protein mục tiêu, bao gồm xóa bỏ tất cả hoặc một phần của gen mục tiêu, bao gồm nhưng không giới hạn ở khung đọc mở của vị trí đích, các chất điều chỉnh phiên mã như các chất xúc tiến của vị trí đích và bất kỳ trình tự axit nucleic điều chỉnh nào khác được định vị 5' hoặc 3' từ khung đọc mở, chèn các codon dừng sớm vào mở khung đọc và các phần chèn hoặc xóa làm thay đổi khung đọc dẫn đến kết thúc sớm sự chuyển đổi. Những đột biến mất đoạn như vậy có thể đạt được bằng cách sử dụng bất kỳ kỹ thuật nào được biết đến với những người có hiểu biết trong lĩnh vực kỹ thuật này. Mức độ giảm của protein mục tiêu hoặc giảm hoạt động của protein mục tiêu cũng có thể đạt được khi đột biến điểm hoặc chèn vào ADN hoặc gen mã hóa protein mục tiêu. Các biến thể đột biến, chèn và mất đoạn của trình tự nucleotit và gen đã bộc lộ có thể được chuẩn bị sẵn sàng bằng các phương pháp mà những người có hiểu biết trong lĩnh vực kỹ thuật này biết rõ. Các kỹ thuật được sử dụng để giảm mức độ và/hoặc giảm hoạt tính của protein mục tiêu có thể bao gồm CRISPR/Cas, TALEN và nucleaza ngón tay kềm. Không vượt quá khả năng của người có hiểu biết trong lĩnh vực kỹ thuật này để tạo ra các đột biến, chèn và thêm bớt có chức năng tương đương với các đột biến cụ thể được bộc lộ ở đây.

Các phương pháp tiếp cận được mô tả ở đây đã được sử dụng để tạo ra đột biến loại bỏ I177L trong ASFV-G có thể được sử dụng trong các phân lập khác nhau của ASFV (chẳng hạn như các phân lập lưu hành ở châu Á, châu Âu hoặc châu Phi), nơi có mặt I177L chức năng. Các phương pháp tiếp cận như vậy có thể khác nhau theo các phương pháp đã biết trong lĩnh vực này, chẳng hạn như sử dụng các dấu hiệu chọn lọc khác nhau có thể chọn virus tái tổ hợp bằng cách tinh chế, chẳng hạn như, nhưng không giới hạn ở, các protein huỳnh quang, các enzym như beta-glucuronidaza hoặc beta-galactosidaza có thể được sử dụng với chất nền tạo màu, và chất tạo lựa chọn thuốc. Các phương pháp như vậy cũng có thể được sử dụng để tạo ra bất kỳ đột biến nào đối với ORF của I177L cũng như các yếu tố điều tiết kiểm soát sự biểu hiện và dịch mã của gen I177L dẫn đến tạo ra protein I177L không có chức năng.

Các đột biến của I177L (và các alen đặc trưng cho chủng có liên quan) trong các chủng và kiểu gen ASFV khác cũng được đề cập theo sáng chế. Các chủng ASFV bao gồm các đột biến tổng hợp trong trình tự axit nucleic biểu hiện ít nhất

80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92 %, 93%, 94%, 95%, 96%, 97%, 98% hoặc 99% đối với SEQ ID NO: 3 được bao hàm trong sáng chế tức thì. Các chủng ASFV bao gồm toàn bộ bộ gen với 95%, 96%, 97%, 98%, 99% hoặc cao hơn đối với SEQ ID NO: 2 cũng được đề cập trong sáng chế.

Sáng chế đề cập thêm về sự kết hợp của gen I177L không có chức năng với các đột biến tái tổ hợp khác. Do đó, không chỉ virus kiểu hoang dã có thể được sửa đổi như được bộc lộ ở đây, mà còn cả các chủng có chứa các đột biến không xảy ra tự nhiên trong các gen hoặc vùng gen khác (xem ví dụ bằng sáng chế Hoa Kỳ số 9.814.771).

Sáng chế đề cập đến rằng ASFV-G Δ I177L sống, giảm độc lực được thiết kế hợp lý như vậy có thể được kết hợp vào các chế phẩm gây miễn dịch để tạo ra vaccin có hiệu quả để bảo vệ động vật, chẳng hạn như lợn, khỏi bệnh ASF lâm sàng khi thử nghiệm với ASFV-G. Do đó, mục tiêu của sáng chế là cung cấp phương pháp bảo vệ động vật chống lại ASFV-G bằng cách sử dụng một lượng hiệu quả vaccin sống giảm độc lực ASFV-G Δ I177L được thiết kế hợp lý. Theo một phương án khác, sáng chế đề cập đến phương pháp tạo ra phản ứng miễn dịch bảo vệ ở động vật, tốt nhất là thuộc họ Suidae (ví dụ, lợn nhà (*Sus scrofa domesticus*), lợn rừng (*Sus scrofa scrofa*), lợn warthog (*Potamochoerus porcus*), lợn lông rậm (*Potamochoerus larvatus*), lợn rừng khổng lồ (*Hylochoerus meinertzhageni*) cũng như lợn hoang). Các phương pháp như vậy thường bao gồm việc sử dụng cho động vật đó một hoặc nhiều chế phẩm và vaccin sinh miễn dịch ASFV được mô tả ở đây.

Một mục tiêu bổ sung theo sáng chế là cung cấp phương pháp phân biệt động vật bị nhiễm ASFV kiểu hoang dã với động vật được tiêm chủng virus tái tổ hợp được mô tả ở đây. Các phương pháp như vậy để phân biệt động vật bị nhiễm bệnh với động vật đã được tiêm phòng (DIVA) có thể được thực hiện bằng các xét nghiệm huyết thanh học để phát hiện sự khác biệt giữa protein I177L kiểu hoang dã và protein I177L đột biến. Ngoài ra, các phương pháp như vậy có thể bao gồm các phương pháp tiếp cận sàng lọc di truyền như khuếch đại PCR và dựa trên phát hiện các sản phẩm khác nhau. Thông thường, các cách tiếp cận như vậy sử dụng một hoặc nhiều bộ môi bao quanh vị trí đột biến và mở rộng cùng một vùng, dẫn đến các sản phẩm có độ dài hoặc trình tự khác nhau.

(Các) chế phẩm sinh miễn dịch theo sáng chế, bất kể các thành phần khác được bao gồm, bao gồm ASFV tái tổ hợp với gen/protein I177L không có chức năng. Các protein I177L theo sáng chế có thể bao gồm toàn bộ SEQ ID NO: 4 và các protein có 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, hoặc nhận dạng cao hơn đối với protein của SEQ ID NO: 4.

Lượng chế phẩm sinh miễn dịch có hiệu quả về mặt sinh học được bộc lộ ở đây có thể thay đổi tùy theo nhiều thông số. Tuy nhiên, nói chung, lượng hữu hiệu trên một đơn vị liều lượng để tiêm bắp có thể là khoảng 10^2 50% liều hấp thụ máu ("HAD₅₀") đến 106 HAD₅₀. Một, hai hoặc nhiều đơn vị liều lượng có thể được sử dụng trong việc thực hành các phương pháp theo sáng chế. Một đơn vị liều lượng có thể dễ dàng được sửa đổi để phù hợp với thể tích hoặc khối lượng mong muốn bằng người có hiểu biết trong lĩnh vực kỹ thuật này. Bất kể thông số đơn vị liều lượng là bao nhiêu, chế phẩm sinh miễn dịch được bộc lộ ở đây có thể được sử dụng với lượng có hiệu quả để tạo ra đáp ứng miễn dịch.

Các mức liều lượng của thành phần hoạt tính trong vaccin được bộc lộ ở đây, có thể được thay đổi theo người có hiểu biết trong lĩnh vực kỹ thuật để đạt được kết quả mong muốn trong một đối tượng hoặc cho mỗi ứng dụng. Do đó, mức liều lượng được lựa chọn có thể phụ thuộc vào nhiều yếu tố bao gồm, nhưng không giới hạn ở công thức, sự kết hợp với các phương pháp điều trị khác, mức độ nghiêm trọng của tình trạng bệnh từ trước, và sự có mặt hoặc không có chất bổ trợ. Theo các phương án tốt hơn, liều lượng tối thiểu của chế phẩm sinh miễn dịch được sử dụng. Việc xác định liều lượng tối thiểu nằm trong khả năng của người có hiểu biết trong lĩnh vực kỹ thuật này.

Vaccin theo sáng chế có thể được điều chế bằng các phương pháp thông thường được sử dụng cho vaccin ASFV sống giảm độc lực có bán trên thị trường. Theo một phương án cụ thể, chất nền nhạy cảm được cấy với đột biến ASFV-G Δ I177L và được nhân giống cho đến khi virus đã nhân lên đến mức mong muốn, sau đó vật liệu chứa ASFV-G Δ I177L được thu hoạch. Sau đó, nguyên liệu thu hoạch có thể được điều chế thành chế phẩm vaccin có đặc tính sinh miễn dịch. Mọi cơ chất có thể hỗ trợ sự nhân lên của virus tái tổ hợp được đề xuất ở đây có thể được sử dụng trong sáng chế, bao gồm cả việc nuôi cấy sơ cấp các đại thực bào máu ngoại vi của lợn hoặc máu từ lợn bị nhiễm bệnh.

Công thức và Truyền

Vaccin được đề xuất ở đây bao gồm một trong các virus tái tổ hợp như đã định nghĩa ở trên ở dạng sống và chất mang hoặc chất pha loãng được dùng thường được sử dụng cho các chế phẩm như vậy. Chất mang bao gồm chất ổn định, chất bảo quản và chất đệm. Các chất ổn định thích hợp bao gồm, ví dụ, SPGA (sucroza, phosphat, glutamat và albumin), cacbohydrat (sorbitol, mannitol, tinh bột, sucroza, dextran, glutamat và glucoza), protein (sữa khô, huyết thanh, albumin, casein), hoặc sự phân hủy các sản phẩm của chúng. Các chất đệm thích hợp bao gồm, ví dụ như phosphat kim loại kiềm. Các chất bảo quản có thể được sử dụng, bao gồm, nhưng không giới hạn ở, thimerosal, merthiolat và gentamixin.

Chất pha loãng bao gồm nước, chất đệm dung dịch nước (ví dụ, dung dịch đệm muối), rượu và polyol (ví dụ, glyxerol).

Trong một số trường hợp, vacxin theo sáng chế cũng chứa hoặc bao gồm một hoặc nhiều chất bổ trợ, bao gồm bất kỳ nguyên liệu nào có trong công thức chế phẩm sinh miễn dịch để tăng cường phản ứng miễn dịch ở người nhận được tạo ra bởi chế phẩm sinh miễn dịch. Trong một số trường hợp, các chất bổ trợ như vậy có thể bao gồm protein các thành phần khác có trong virus tái tổ hợp. Các chất bổ trợ khác có thể gồm: thành phần bổ sung của chế phẩm sinh miễn dịch, và bao gồm các loại như muối nhôm (phèn), nhũ tương dầu, saponin, phức hợp kích thích miễn dịch (ISCOM), liposom, vi hạt, copolyme khối không ion, polysaccharit dẫn xuất, xytokin và nhiều loại dẫn xuất vi khuẩn. Bất kỳ chất bổ trợ liên quan nào đã biết trong lĩnh vực này đều có thể được sử dụng trong việc thực hành các sáng chế được bộc lộ ở đây. Các yếu tố ảnh hưởng đến việc lựa chọn một chất bổ trợ bao gồm loài động vật, mầm bệnh cụ thể, kháng nguyên, đường tiêm chủng và loại miễn dịch cần thiết và có thể dễ dàng xác định bằng người có hiểu biết trong lĩnh vực kỹ thuật này.

Chế phẩm sinh miễn dịch theo sáng chế cũng có thể bao gồm chất mang ngoài virus tái tổ hợp. Chất mang được sử dụng trong thực hành chế phẩm sinh miễn dịch được đề xuất ở đây có thể là chất bất kỳ đã biết trong lĩnh vực này và có thể ở dạng lỏng, rắn, bán rắn hoặc gel. Loại công thức có thể được sửa đổi tùy thuộc vào đường sử dụng kháng nguyên. Tốt hơn là chất mang không độc hại cho người nhận. Người có hiểu biết trong lĩnh vực kỹ thuật này có thể dễ dàng chọn những chất mang như vậy để áp dụng cho động vật nhận như gia cầm.

Sáng chế đề cập đến các chế phẩm sinh miễn dịch để đưa ASFV tái tổ hợp thiếu gen/protein I177L chức năng trong chế phẩm có chứa tối thiểu virus tái tổ hợp vào mục tiêu (ví dụ, lợn). Do đó, các chế phẩm được đề cập ở đây có thể được sử dụng để tạo ra khả năng miễn dịch hoặc đề kháng với bệnh ASFV.

Các loại vacxin được đề cập ở đây có thể được sử dụng bằng cách tiêm bắp, tiêm dưới da, tiêm trong mũi hoặc tiêm với một lượng có hiệu quả để bảo vệ động vật chống lại sự thách thức của một chủng độc lực của ASFV. Vacxin có thể được dùng bằng đường uống, thông qua việc uống trực tiếp vào miệng, được định lượng trong nước uống hoặc thông qua hệ thống phân phối môi. Số lượng hiệu quả của virus tái tổ hợp có thể thay đổi tùy theo các thông số được xem xét bởi người có hiểu biết trong lĩnh vực kỹ thuật này. Lượng hữu hiệu có thể được xác định bằng thực nghiệm khi cần thiết bởi người có hiểu biết trong lĩnh vực kỹ thuật này bằng cách thực hiện theo bất kỳ phương pháp nào đã biết hoặc hướng dẫn được đề cập trong các ví dụ ở đây.

Sau khi được mô tả chung về sáng chế này, điều tương tự sẽ được hiểu rõ hơn bằng cách tham chiếu đến một số ví dụ cụ thể, được bao gồm ở đây để minh họa thêm cho sáng chế và không nhằm giới hạn phạm vi của sáng chế như được xác định bởi các yêu cầu bảo hộ.

Ví dụ thực hiện sáng chế

Ví dụ 1

Nuôi cấy tế bào và virus.

Nuôi cấy tế bào đại thực bào lợn sơ cấp được chuẩn bị từ máu lợn đã khử chất béo như đã mô tả trước đây (Zsak và cộng sự, J.Virol., (1998) 72: 1028-35). Nói một cách tóm tắt, máu lợn được xử lý bằng heparin được ủ ở 37°C trong 1 giờ để cho phép lắng phần hồng cầu. Bạch cầu đơn nhân được phân tách bằng cách tuyển nổi trên gradient mật độ (trọng lượng riêng, 1,079) Ficoll-Paque (Pharmacia, Piscataway, N.J.). Phần tế bào đơn nhân/đại thực bào được nuôi cấy trong bình nuôi cấy mô bằng đĩa Primaria nhựa (Falcon; Becton Dickinson Labware, Franklin Lakes, N.J.) có chứa môi trường đại thực bào, bao gồm Môi trường RPMI 1640 (Life Technologies, Grand Island, NY) với 30% L929 chất nổi và 20% huyết thanh bò thai (HI-FBS, Thermo Scientific, Waltham, MA) trong 48 giờ ở 37°C dưới 5% CO₂. Các tế bào kết dính được tách ra khỏi nhựa bằng cách sử dụng 10 mM EDTA trong dung dịch muối đệm phosphat (PBS) và sau đó được gửi lại vào đĩa Primaria T25, 6 hoặc 96 đĩa giếng với mật độ 5x10⁶ tế bào/ml để sử dụng trong các xét nghiệm 24 giờ sau đó.

Chuẩn độ virus được thực hiện trên nuôi cấy tế bào đại thực bào lợn sơ cấp trong đĩa 96 giếng. Việc pha loãng và nuôi cấy virus được thực hiện bằng cách sử dụng môi trường đại thực bào. Sự hiện diện của virus được đánh giá bằng hấp thụ máu (HA) và hiệu giá virus được tính bằng phương pháp Reed và Muench (Amer. J. Hygiene, (1938) 27: 493-497).

ASFV Georgia (ASFV-G) được sử dụng cho nghiên cứu này là một chủng phân lập trên thực địa do Tiến sĩ Nino Vepkhvadze, từ Phòng thí nghiệm của Bộ Nông nghiệp (LMA) ở Tbilisi, Cộng hòa Georgia cung cấp.

Ví dụ 2

Cấu tạo ASFV-G ΔI177L tái tổ hợp

ASFV tái tổ hợp được tạo ra bằng cách tái tổ hợp tương đồng tuần tự giữa bộ gen ASFV của bố mẹ và các vector chuyển giao tái tổ hợp trong các quy trình lây nhiễm và chuyển nhiễm bằng cách sử dụng nuôi cấy tế bào đại thực bào lợn (Neilan và cộng sự, Virol., (2004) 319: 337-42; Zsak và cộng sự, như trên). Vector

chuyển tái tổ hợp (p72GUS Δ I177L) chứa các vùng gen bên sườn bao gồm các phần của ánh xạ I177L ở bên trái (1 kbp) và bên phải (1 kbp) của gen và một băng gen phóng viên chứa gen mCherry với trình tự khởi đầu gen muợn ASFV p72, p72mCherry là đã sử dụng. Cấu trúc này tạo ra sự mất đoạn 112 nucleotit trong ORF I177L (gốc axit amin từ 112 đến 150) (Hình 1). Vectơ chuyển tái tổ hợp p72mCherry Δ I177L thu được bằng cách tổng hợp ADN (Epoch Biosciences, Bothwell, WA, USA). Nuôi cấy tế bào đại thực bào bị nhiễm ASFV-G và được truyền p72mCherry Δ I177L. Các virus tái tổ hợp đại diện cho các mảng sơ cấp độc lập đã được tinh sạch để đồng nhất bằng các vòng tinh sạch liên tiếp của xét nghiệm mảng bám. Virus tái tổ hợp thu được sau 14 giai đoạn thanh lọc mảng bám liên tiếp trên các lớp đơn của nuôi cấy tế bào đại thực bào lợn sơ cấp.

Ví dụ 3

Phân tích trình tự bộ gen đầy đủ của ASFV-G Δ I177L liên quan đến gen ASFV-G bố mẹ.

Để đánh giá độ chính xác của việc chỉnh sửa gen và tính toàn vẹn của bộ gen của virus tái tổ hợp, trình tự bộ gen đầy đủ của ASFV-G Δ I177L và gen ASFV-G bố mẹ được thu thập bằng cách sử dụng Giải trình tự thế hệ tiếp theo (NGS) và so sánh. Bước đầu tiên, so sánh bộ gen với chiều dài đầy đủ giữa chủng ASFV-G trong phòng thí nghiệm của bố mẹ được sử dụng để tạo ra virus đột biến ASFV-G Δ I177L và virus ASFV Georgia 2007/1 ban đầu (Chapman và cộng sự, *Emerg. Infect. Dis.*, (2001) 17: 599-605; số tham gia GenBank FR682468) đã được thực hiện. ADN ASFV được lấy từ tế bào chất của các tế bào bị nhiễm bằng phương pháp Trizol (Life Technologies, Grand Island, NY, USA). Nồng độ ADN được xác định bằng cách sử dụng bộ xét nghiệm Qubit® dsADN HS (Life Technologies) và đọc trên Thiết bị lắng trong Qubit® 2 (Life Technologies). Trong bản tóm tắt, ADN của virus được cắt bằng cách sử dụng các phản ứng enzym được đánh giá về sự phân bố của phân mảnh kích thước, sau đó thất các mã vạch xác định bằng trình tự tiếp hợp được thêm vào các đoạn ADN. Sử dụng Pippin Prep™ (Sage Science, Beverly, MA), phạm vi kích thước yêu cầu của thư viện đã được thu thập và chuẩn hóa. Sau đó, chủ đơn sử dụng thư viện ADN này để giải trình tự NGS bằng NextSeq (Illumina, San Diego, CA) theo giao thức sản xuất. Phân tích trình tự được thực hiện bằng phần mềm CLC Genomics Workbench (CLCBio, Waltham, MA)

Sự khác biệt sau đây đã được quan sát thấy giữa hai loại virus này (vị trí nucleotit được cung cấp dựa trên ASFV Georgia 2007/1, số tham gia GenBank FR682468?): (I) chèn ba nucleotit, T ở vị trí 433, A ở vị trí 441 ở dạng không mã hóa phân đoạn của bộ gen và một A ở vị trí 174954 trong gen I177L, khiến I177L

hợp nhất với ORF ASFV_G_ACD_01760, nucleotit bổ sung này cho phép ORF I117L giống với một gen có chiều dài đầy đủ tương tự như các chủng phân lập khác, như với A bổ sung, có không phải là codon dừng sớm và đột biến ngoài khung như được mô tả trong bộ gen tham chiếu; (ii) mất hai nucleotit, T ở vị trí 1602 và T ở vị trí 1603 trong ORF gen MGF 360-1L dẫn đến dịch chuyển khung; (iii) sự mất đoạn nucleotit, T ở vị trí 1620 trong ORF của gen MGF 360-1L dẫn đến hiện tượng lệch khung; (iv) đột biến nucleotit, A thành G ở vị trí 97391 dẫn đến đột biến câm trong ORF B438L; (v) đột biến nucleotit, C thành G ở vị trí 166192 dẫn đến sự thay thế gốc (Ala thành Pro) ở vị trí gốc 85 trong ORF E199L; và (vi) một đoạn chèn nucleotit, ở vị trí 183303, một đoạn không mã hóa của bộ gen.

Để xác định xem liệu virus tái tổ hợp có nhận được những thay đổi di truyền bổ sung từ dòng bố mẹ hay không, một phép so sánh bộ gen có chiều dài đầy đủ giữa ASFV-G Δ I177L và gen ASFV-G bố mẹ đã được thực hiện. Các tổ hợp trình tự ADN của ASFV-G Δ I177L và ASFV-G bộc lộ sự mất đoạn 112 nucleotit trong gen I177L tương ứng với sửa đổi được đưa vào. Trình tự đồng thuận của bộ gen ASFV-G Δ I177L cho thấy sự chèn 3944 nucleotit trong gen I177L tương ứng với trình tự cat-xet p72-mcherry được đưa vào để tạo ra sự xóa 112 nucleotit trong gen mục tiêu. Bên cạnh việc chèn cat-xet, không có sự khác biệt nào được quan sát thấy giữa bộ gen ASFV-G Δ I177L và ASFV-G. Tóm lại, virus ASFV-G Δ I177L không tích lũy bất kỳ đột biến đáng kể nào trong quá trình tái tổ hợp tương đồng và thanh lọc mảng bám.

Ví dụ 4

Đánh giá độc tố ASFV-G Δ I177L ở lợn.

Các thí nghiệm trên động vật được thực hiện trong điều kiện an toàn sinh học cấp độ 3 trong các cơ sở động vật tại PIADC theo một quy trình đã được Cơ quan Ủy ban Chăm sóc và Sử dụng Động vật phê duyệt.

ASFV-G Δ I177L được đánh giá về kiểu hình độc lực của nó so với virus ASFV-G bố mẹ có độc lực bằng cách sử dụng lợn giống thương phẩm nặng 36,29-40,82 kg. Năm con lợn được tiêm bắp (IM) với 10^2 , 10^4 , 10^6 HAD₅₀ của ASFV-G Δ I177L hoặc với 10^2 HAD₅₀ của virus ASFV-G. Các dấu hiệu lâm sàng (chán ăn, trầm cảm, sốt, đổi màu da tím, dáng đi loạng choạng, tiêu chảy và ho) và sự thay đổi nhiệt độ cơ thể được ghi lại hàng ngày trong suốt thí nghiệm. Trong các thí nghiệm bảo vệ, động vật được cấy IM với 10^2 , 10^4 , 10^6 HAD₅₀ của ASFV-G Δ I177L và IM được cấy 28 ngày đã thử thách với 10^2 HAD₅₀ của dòng ASFV-Georgia 2007 bố mẹ độc lực. Sự hiện diện của các dấu hiệu lâm sàng liên quan đến bệnh đã được đánh giá như mô tả trước đó.

Tất cả những con lợn được cấy qua IM với 10^4 HAD₅₀ của ASFV-G đều biểu hiện nhiệt độ cơ thể tăng lên ($> 38,89^\circ\text{C}$) từ 3 đến 4 ngày sau khi nhiễm bệnh. Lợn có các dấu hiệu lâm sàng liên quan đến bệnh bao gồm biếng ăn, suy nhược, da tím tái, đáng đi loạng choạng và tiêu chảy (Bảng 1). Các dấu hiệu của bệnh tiến triển nặng hơn theo thời gian và động vật chết hoặc bị tử vong cực độ vào ngày thứ 7 hoặc 9 sau khi nhiễm bệnh. Ngược lại, những con vật được cấy qua IM với 10^2 , 10^4 , hoặc 10^6 HAD₅₀ của ASFV-G Δ I177L không có bất kỳ dấu hiệu nào của bệnh lâm sàng trong toàn bộ thời gian quan sát (21 ngày). Do đó, việc xóa bỏ gen I177L đã tạo ra sự suy giảm hoàn toàn của ASFV-G độc lực của bố mẹ. Tất cả các động vật trong nhóm được tiêm vacxin Mock đã được cho chết vì lý do nhân đạo theo giao thức IACUC tương ứng.

Bảng 1: Tỷ lệ sống và phản ứng sốt của lợn sau khi nhiễm 10^2 HAD₅₀ của ASFV-G- Δ I177L hoặc ASFV-G bố mẹ.

Virut	Số lượng sống/ tổng số	Thời gian trung bình để chết (ngày $\pm$ SD)	Sốt		
			Số ngày bắt đầu (ngày $\pm$ SD)	Khoảng thời gian Số ngày (ngày $\pm$ SD)	Nhiệt độ tối đa hàng ngày ($^\circ\text{C} \pm$ SD)
ASFV-G	0/5	4,8 (0,84)	4,8 (0,84)	0,8 (0,84)	40,67 (0,6)
ASFV-G- Δ I177L 10^2 HAD ₅₀	5/5	-	-	-	39,39 (0,5)
ASFV-G- Δ I177L 10^4 HAD ₅₀	5/5	-	-	-	39,33 (0,57)
ASFV-G- Δ I177L 10^6 HAD ₅₀	5/5	-	-	-	39,33 (0,49)

Các con vật bị nhiễm ASFV-G có hiệu giá đồng nhất cao dự kiến ($10^{7,5}$ - $10^{8,5}$ HAD₅₀/ml) vào ngày thứ 4 pi, tăng (khoảng $110^{8,5}$ HAD₅₀/ml/ml) vào ngày thứ 7 khi tất cả các con vật bị chết. Ngược lại, ASFV-G- Δ I177L cho thấy một mô hình khác với lượng viremia thấp ($10^{1,8}$ - $10^{2,3}$ HAD₅₀/ml) ở ngày 4 pi, đạt giá trị đỉnh ($10^{3,8}$ - $10^{7,5}$ HAD₅₀/ml) vào ngày 11 pi và sau đó giảm hiệu giá ($10^{2,3}$ - 10^4 HAD₅₀/ml) cho đến ngày 28 pi (Hình 3). Cần lưu ý rằng một trong năm con vật được cấy ASFV-G- Δ I177L cho thấy lượng viremia thấp hơn đáng kể (thấp hơn 1.000 đến 10.000 lần tùy thuộc vào thời điểm được xem xét) so với giá trị virus trong máu trung bình của các con khác trong nhóm. Do đó, việc loại bỏ gen I177L tạo ra sự suy giảm hoàn toàn của virus ASFV-G độc lực cao của bố mẹ khi được cấy ở liều lượng thấp, với những con bị nhiễm bệnh biểu hiện virus kéo dài với giá trị tương đối thấp.

Ví dụ 5

Hiệu quả bảo vệ của ASFV-G Δ I177L trong việc chống lại thách thức với ASFV-G bố mẹ.

Vì lợn được cấy qua IM với 10^2 HAD₅₀- 10^6 HAD₅₀ của ASFV-G Δ I177L sống sót sau nhiễm trùng mà không có dấu hiệu của bệnh, các nhóm động vật ($n = 5$) được tiêm 10^2 HAD₅₀- 10^6 HAD₅₀ của ASFV-G Δ I177L đã được thử nghiệm qua IM với 10^2 HAD₅₀ của ASFV-G bố mẹ ở ngày 28 sau khi cấy (thử thách tương đồng). Năm loại động vật chưa quen thí nghiệm đã được thử thách bằng cách sử dụng cùng một con đường và liều lượng phục vụ như một nhóm đối chứng không được tiêm chủng/ thử thách. Tất cả các động vật được tiêm vaccin IM với 10^2 HAD₅₀ của ASFV-G Δ I177L và thử thách IM 28 ngày sau với 10^2 HAD₅₀ của virus ASFV-G. Tất cả các động vật trong nhóm được tiêm vaccin Mock đã được cho chết vì lý do nhân đạo theo giao thức IACUC tương ứng. Tất cả các động vật trong nhóm được tiêm chủng ASFV-G Δ I177L vẫn bình thường về mặt lâm sàng trong thời gian quan sát 21 ngày sau thử thách.

Năm động vật được cấy và thử thách ASFV-G Δ I177L vẫn hoàn toàn không có triệu chứng trong suốt thời gian quan sát (21 ngày) (Bảng 2). Tất cả các động vật trong nhóm đối chứng được tiêm/thử thách mô phỏng đều phát triển bệnh với diễn biến lâm sàng tương tự như được quan sát thấy ở động vật được tiêm 10^2 HAD₅₀ của ASFV-G (xem ở trên). Do đó, ASFV-G Δ I177L có thể tạo ra sự bảo vệ chống lại sự biểu hiện của bệnh lâm sàng khi thách thức với virus bố mẹ có độc lực cao.

Bảng 2: Khả năng sống sót của lợn và phản ứng với sốt ở động vật thử thách với virus ASFV-G ở 28 ngày sau khi nhiễm ASFV-G- Δ I177L.

Virus	Số lượng sống sót/tổng số	Thời gian trung bình để chết (ngày + SD)	Sốt		
			Số ngày bắt đầu (ngày $\pm$ SD)	Khoảng thời gian Số ngày (ngày + SD)	Nhiệt độ tối đa hàng ngày ($^{\circ}\text{C} \pm \text{SD}$)
Mock	0/5	5,6 (0,55) (1)	4,2 (0,84)	1,4 (0,88)	40,89 (0,78)
ASFV-G- Δ I177L 10^2 HAD ₅₀	10/10	-	-	-	39,28 (0,68)
ASFV-G- Δ I177L 10^4 HAD ₅₀	5/5	-	-	-	39,39 (0,37)
ASFV-G- Δ I177L 10^6 HAD ₅₀	5/5	-	-	-	39,44 (0,43)

Phân tích viremia ở động vật bị nhiễm ASFV-G cho thấy hiệu giá cao dự kiến ($10^{7.3} - 10^{8.3}$ HAD₅₀/m) vào ngày thứ 4 pi, tăng (trung bình $10^{8.5}$ HAD₅₀/ml) pi vào ngày thứ 7 khi tất cả động vật được cho chết. Sau thử thách, không có con vật nào bị nhiễm ASFV-G-ΔI177L có các viremia với giá trị cao hơn những con có ở thử thách và giá trị virus trong máu giảm dần cho đến khi kết thúc giai đoạn thử nghiệm (21 ngày sau thử thách) khi, quan trọng là không có virus lưu hành nào có thể được phát hiện trong máu của bất kỳ động vật nào trong số này (Hình 5).

Tóm lại, ở đây chủ đơn đưa ra bằng chứng cho thấy việc xóa gen I177L làm thay đổi đáng kể độc lực của ASFV-G, tạo ra một loại virus hoàn toàn giảm độc lực có tên là ASFV-G ΔI177L. Động vật được miễn dịch với ASFV-G ΔI177L đã được bảo vệ chống lại thách thức với ASFV-G độc lực của bố mẹ.

Ví dụ 6

Khả năng phát triển của ASFV-G-I117L trong đại thực bào lợn

Các đặc điểm tăng trưởng của thí nghiệm trong ống nghiệm của ASFV-G-ΔI177L được đánh giá trong nuôi cấy tế bào đại thực bào lợn sơ cấp, tế bào chính được ASFV nhắm mục tiêu trong quá trình lây nhiễm ở lợn và so sánh với ASFV-G của bố mẹ trong các đường cong tăng trưởng nhiều bước (Hình 2). Nuôi cấy tế bào bị nhiễm ở MOI là 0,01 và các mẫu được thu thập ở 2, 24, 48, 72 và 96 giờ sau khi nhiễm (hpi). Kết quả chứng minh rằng ASFV-G-ΔI177L hiển thị động năng tăng trưởng giảm đáng kể khi so sánh với ASFV-G bố mẹ. Năng suất ASFV-G-ΔI177L thấp hơn khoảng 100 đến 1.000 lần so với sản lượng của ASFV-G tùy thuộc vào thời điểm được xem xét.

Do đó, việc loại bỏ gen I177L đã làm giảm đáng kể khả năng của ASFV-G-ΔI177L, so với chủng phân lập ASFV-G bố mẹ, để sao chép trong ống nghiệm trong nuôi cấy tế bào đại thực bào lợn sơ cấp.

Ví dụ 7

Động vật bị nhiễm ASFV-G-ΔI117L không thải virus vaccine

Trong ví dụ trên, khi các nhóm khác nhau gồm năm con lợn bị nhiễm IM 10^2 , 10^4 , hoặc 10^6 HAD₅₀ của ASFV-G-ΔI177L, một con vật bị nhiễm giả đang chung sống trong mỗi nhóm với tư cách là lính canh để phát hiện ra virus tiềm ẩn từ động vật bị nhiễm bệnh. Tất cả các động vật trọng điểm vẫn bình thường về mặt lâm sàng (Hình 4). Không có virus nào được phát hiện trong bất kỳ mẫu nào thu được từ động vật trọng điểm (tất cả các mốc thời gian lấy mẫu máu cũng như các mẫu amidan và lá lách thu được ở 28 ngày pi), cho thấy rằng động vật bị nhiễm ASFV-G-ΔI177L không có khả năng thải đủ virus để lây nhiễm cho những con lợn chưa thành niên trong 28 ngày, khoảng thời gian chung sống tương đối dài.

Tóm lại, một con vật không được tiêm phòng đến với những con vật đã được tiêm phòng trong 28 ngày không có bất kỳ triệu chứng lâm sàng nào và tất cả các mẫu máu, amidan và lá lách được lấy mẫu đều âm tính với virus vắc xin, cho thấy virus vắc xin không thể chuyển sang một loài không phải động vật đã được tiêm phòng.

Ví dụ 8

Phản ứng kháng thể vật chủ ở động vật bị nhiễm ASFV-G-ΔI177L

Tất cả các động vật bị nhiễm ASFV-G-ΔI177L, bất kể liều lượng virus nhận được đều sở hữu hiệu giá cao như nhau của các kháng thể kháng ASFV lưu hành (Hình 6). Đáp ứng kháng thể, qua trung gian của các lớp kháng thể IgM và IgG, bắt đầu được phát hiện ở cả ba nhóm vào ngày 12 pi. Đáp ứng kháng thể qua trung gian IgM biến mất ở tất cả các động vật vào ngày 21 pi, trong khi đáp ứng qua trung gian IgG duy trì ở mức cao với dao động tối thiểu cho đến ngày 28 pi mà không có sự khác biệt đáng kể giữa các động vật trong ba nhóm được cấy ASFV-G-ΔI177L. Do đó, có mối tương quan chặt chẽ giữa sự hiện diện của kháng thể chống ASFV tại thời điểm thử thách và khả năng bảo vệ. Cần lưu ý rằng không có kháng thể nào được phát hiện trong bất kỳ mẫu huyết thanh nào thu được từ động vật trọng điểm ngoại trừ một mẫu ở 28 ngày sau khi nhiễm ASFV ở nhóm nhận 10^6 HAD₅₀ của G-ΔI177L nơi hiệu giá kháng thể thấp được quan sát thấy (Hình 6).

Ví dụ 9

Cảm ứng miễn dịch vô trùng

Sử dụng PCR thời gian thực cụ thể I177L để chỉ phát hiện đặc biệt virus thử thách (cho phép phát hiện khoảng 10 HAD₅₀) tất cả các mẫu máu được xét nghiệm âm tính với sự hiện diện của virus thử thách. Hơn nữa, các mẫu amidan và lá lách được lấy từ tất cả các động vật vào cuối giai đoạn quan sát (21 ngày sau thử thách) và được kiểm tra sự hiện diện của virus bằng cách phân lập virus trong nuôi cấy đại thực bào lợn. Hầu hết các động vật trong mỗi nhóm cho thấy sự hiện diện của virus truyền nhiễm hoặc ở amidan hoặc lá lách (dữ liệu không được hiển thị). Tất cả các mẫu dương tính sau đó được đánh giá bằng cách sử dụng PCR thời gian thực cụ thể I177L phát hiện sự hiện diện của virus thử thách chỉ trong một lá lách của một trong những con vật ban đầu bị nhiễm 10^2 HAD₅₀/ml ASFV-G-ΔI177L. Những kết quả này cho thấy rằng không có sự nhân lên của virus thử thách ở tất cả các động vật bị nhiễm bệnh nhận 10^4 HAD₅₀/ml hoặc cao hơn và hầu hết các động vật nhận được 10^2 HAD₅₀/ml ASFV-G-ΔI177L.

Tóm lại, miễn dịch vô trùng (miễn dịch không cho phép sao chép virus thách thức) đã đạt được một phần ở động vật được tiêm vắc xin ASFV-G-ΔI177L 10^2

HAD₅₀/ml và đạt được hoàn toàn ở liều 10⁴ HAD₅₀/ml hoặc 10⁶ HAD₅₀/ml ASFV-G-ΔI177L.

Mặc dù sáng chế đã được mô tả có tham chiếu đến các chi tiết của các phương án được minh họa, nhưng những chi tiết này không nhằm giới hạn phạm vi của sáng chế như được xác định trong các yêu cầu bảo hộ. Các phương án theo sáng chế trong đó xác nhận quyền sở hữu hoặc đặc quyền độc quyền được xác định như dưới đây.

DANH MỤC TRÌNH TỰ

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 BORCA, MANUEL V.

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Thr Pro Val Leu Phe Ile Lys Phe Gln Leu Ile Ala Ala Asp Asn Tyr
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Gln Ala Ile Thr Trp Lys Asp Gly Ile Leu Asn Tyr Glu Lys Ile Asp
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Gln Pro Thr Pro Leu Tyr Leu Ser Val Asn Gly Leu Ile Phe Asp Cys
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Ala Lys Leu Gln Pro Leu Thr Thr Lys Ser Asn Val Thr Ser Gly Asp
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Lys Val Val His Ile Gly Gln Thr Phe Glu Tyr Asn Asn Leu Leu Met
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Trp Lys Val Asn Asp Gln Gly Phe Leu Asn Ile Ser Val Thr Gly Thr
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Lys Phe Asn Leu Ile Ala Ile Thr Gly Lys Leu Gly Phe Tyr Thr Asp
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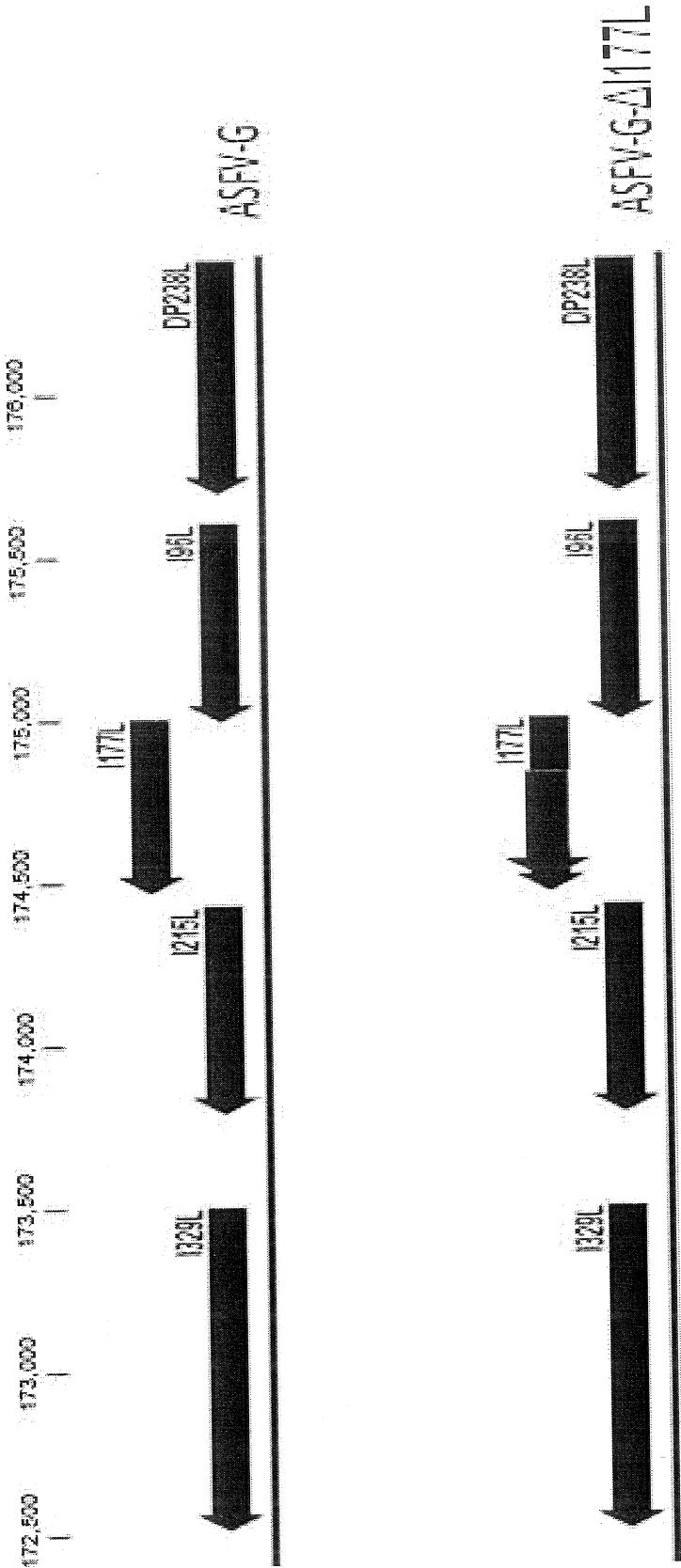
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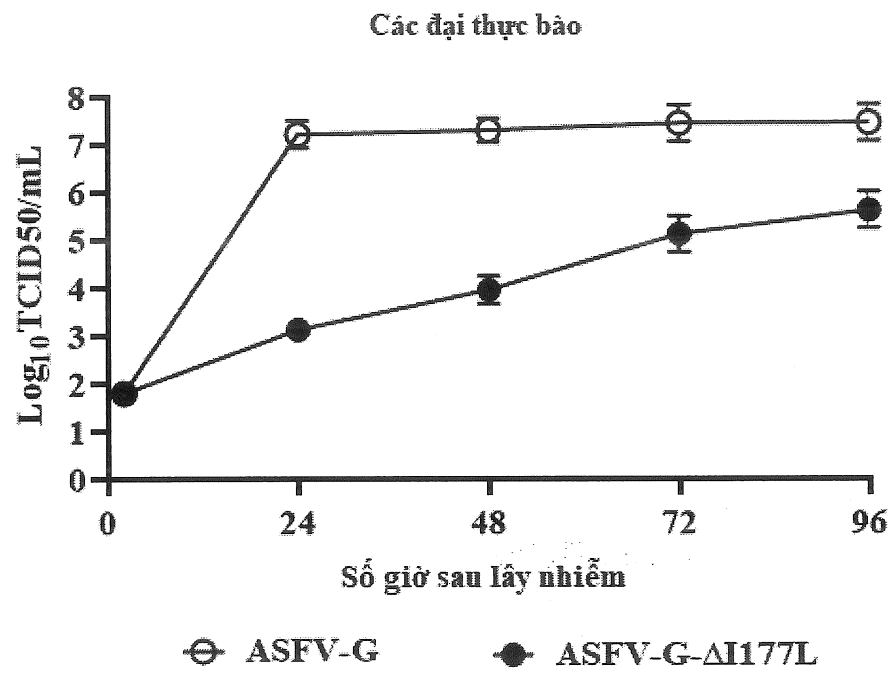
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1. Virut biến đổi gen, trong đó bộ gen của virut bao gồm bộ gen của virut giống ít nhất 95% với SEQ ID NO: 2.
2. Virut theo điểm 1, trong đó bộ gen của virut bao gồm SEQ ID NO: 2.
3. Chế phẩm vaccin chống lại virut gây bệnh dịch tả lợn Châu Phi (ASFV), bao gồm virut biến đổi gen theo điểm 1, trong đó ASFV là phân lập ASFV-Georgia 2007 (ASFV-G).
4. Virut đột biến ASFV tái tổ hợp, bao gồm đột biến tổng hợp trong khung đọc mở I177L hoặc trong yếu tố điều hòa kiểm soát sự biểu hiện của protein I177L, dẫn đến gen I177L trong bộ gen không có chức năng.
5. Virut tái tổ hợp theo điểm 4, trong đó ASFV đột biến bao gồm bộ gen giống ít nhất 99% với SEQ ID NO: 2.
6. Virut theo điểm 1, trong đó bộ gen của virut bao gồm bộ gen của virut giống ít nhất 99% với SEQ ID NO: 2.

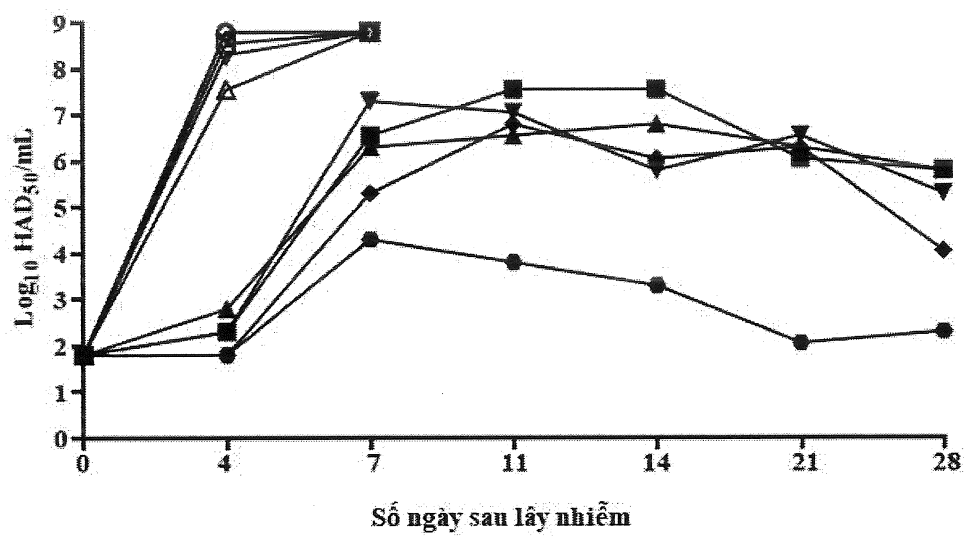


Mũi tên đỏ = Cat-xet p72mCherry

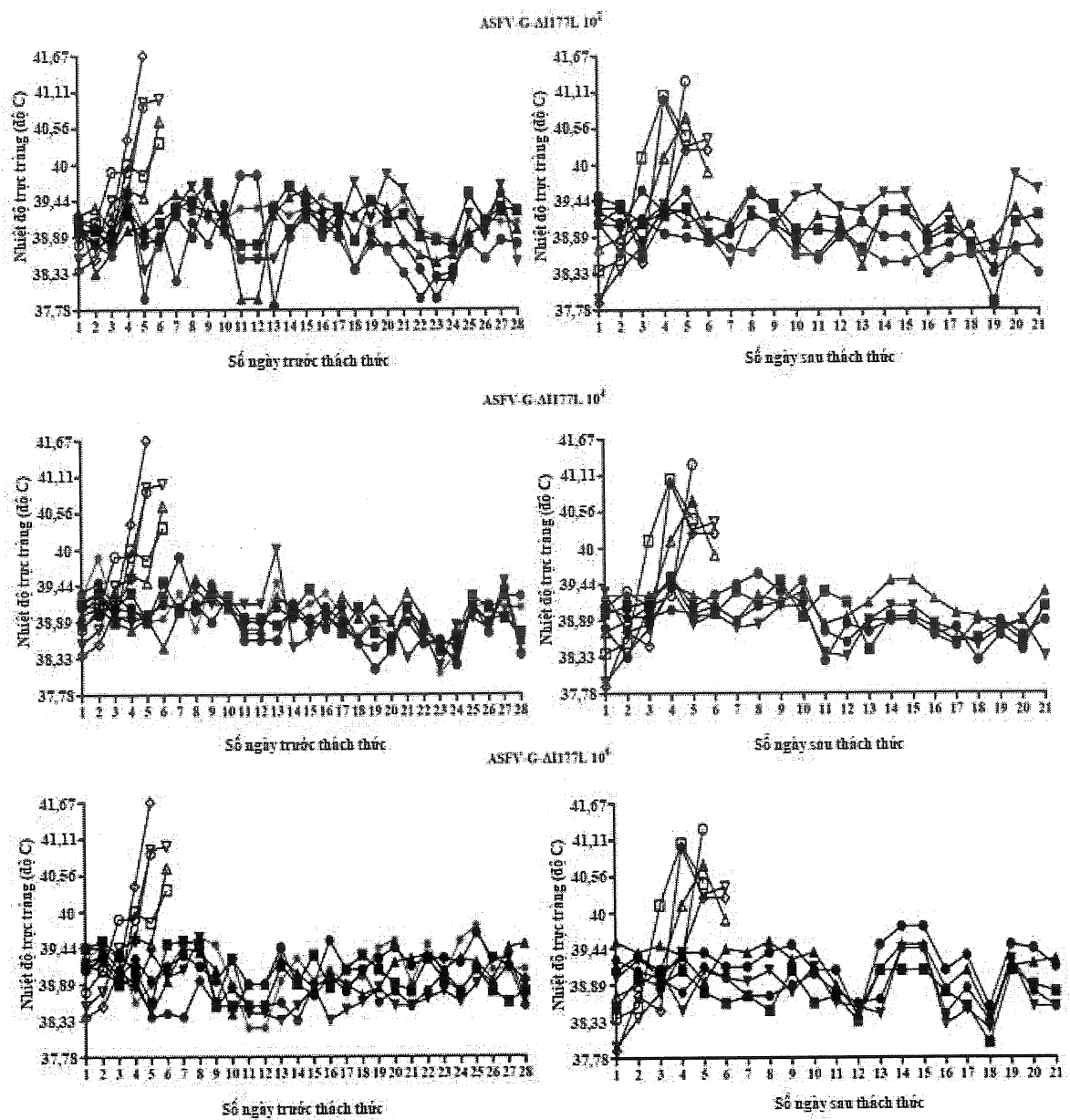
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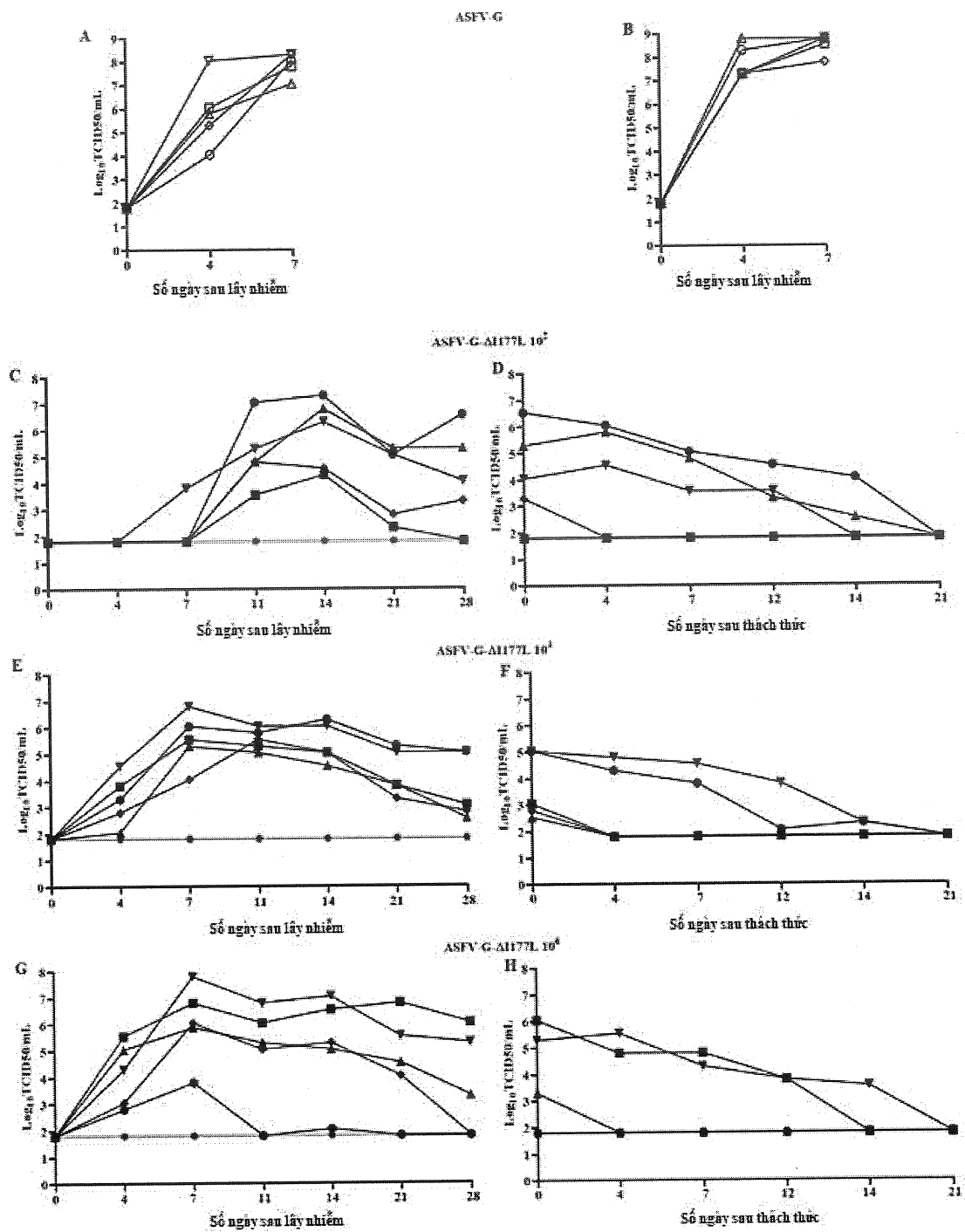
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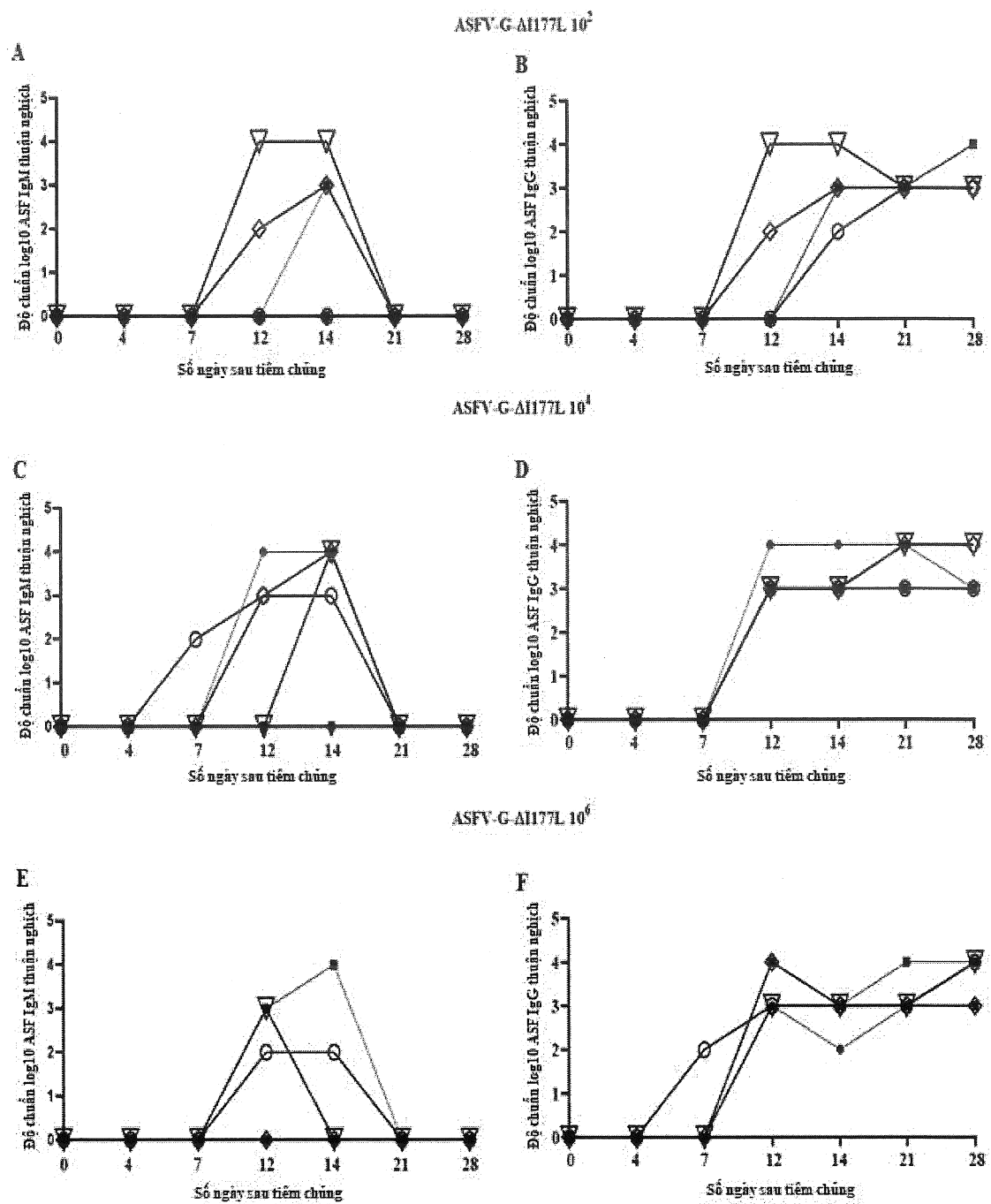
HÌNH 3



HÌNH 4



HÌNH 5



HÌNH 6