Revisiting Jenner's mysteries, the role of the Beaugency lymph in the evolutionary path of ancient smallpox vaccines

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In 1796, Edward Jenner developed the smallpox vaccine consisting of pustular material obtained from lesions on cows affected by so-called cow-pox. The disease, caused by cowpox virus, confers crossprotection against smallpox. However, historical evidence suggests that Jenner might have used vaccinia virus or even horsepox virus instead of cowpox virus. Mysteries surrounding the origin and nature of the smallpox vaccine persisted during the 19th century, a period of intense exchange of vaccine strains, including the Beaugency lymph. This lymph was obtained from spontaneous cases of cow-pox in France in 1866 and then distributed worldwide. A detailed Historical Review of the distribution of the Beaugency lymph supports recent genetic analyses of extant vaccine strains, suggesting the lymph was probably a vaccinia strain or a horsepox-like virus. This Review is a historical investigation that revisits the mysteries of the smallpox vaccine and reveals an intricate evolutionary relationship of extant vaccinia strains.

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Introduction

Smallpox is a dreadful disease that has killed millions of people over the centuries. The disease was eradicated in 1977 thanks to an intense surveillance programme done by WHO and worldwide vaccination that used one of the most efficient vaccines ever developed. The smallpox vaccine was discovered by Edward Jenner (1749–1823) in 1796 and is a hallmark in the history of infectious diseases.[1–3]

Variola virus, the causative agent of smallpox, is a poxvirus of the genus Orthopoxvirus. Cowpox virus is also an orthopoxvirus, and supposedly the one used by Jenner in 1796.[1,4] However, mysteries surround the history of the smallpox vaccine, particularly the nature of the vaccine materials that Jenner and others propagated during the 18th and 19th centuries. Historical evidence, associated with recent scientific studies, suggests that the orthopoxviruses vaccinia or horsepox, but not cowpox virus, were most likely the actual vaccine sources. These sources also include the famous worldwide-distributed Beaugency lymph, which certainly contributed to the genetic content of several modern vaccine strains. Therefore, in this Review, I use the old names cow-pox and horse-pox to refer to uncertain cases of the disease that occurred between this period—eg, pustular diseases in cows in the 18th and 19th centuries are referred to by Jenner and others as cow-pox. The same is true for horse-pox in horses. I used the old names to suggest that they might not have been caused by cowpox virus or horsepox virus.

Jenner and the smallpox vaccine

Jenner was a British physician who worked in Berkeley, Gloucestershire, UK. In 1796, he decided to investigate previous anecdotal observations that milkers who acquired so-called cow-pox disease from infected cows were protected against smallpox.[5–7] His renowned experiment consisted of taking pustular material from cow-pox lesions on the hands of the milker Sarah Nelmes and inoculating the 8-year-old James Phipps in the arm (figure 1). 6 weeks later, Jenner challenged the boy with pustular material from a smallpox lesion but James did not develop smallpox.[5] Jenner did not understand exactly how protection against smallpox was achieved. Scientists and physicians now know that infection with orthopoxvirus confers crossimmunity against subsequent infection with another orthopoxvirus.[1,4]

In 1800, Jenner's friend Richard Dunning referred to the procedure done by Jenner as vaccination (from Latin *vacca*, cow), whereas the pustular material, or lymph, was referred to as vaccine, cowpox virus, or vaccinia virus interchangeably, as if both viruses were the same thing.[8–10] Nonetheless, in 1939, British researcher Allan Downie (1901–88) showed that vaccinia virus (meaning the smallpox vaccine) and a strain of cowpox virus that had been isolated from naturally infected cows had different biochemical properties and thus could not be the same virus.[11] This finding was later confirmed, and full-genome sequencing now shows unequivocally that vaccinia and cowpox viruses are different orthopoxviruses.[12,13]

If vaccinia virus had been introduced randomly over the course of vaccine manufacture in the 18th or 19th centuries, one should expect to find 20th century vaccine strains containing either virus (cowpox or vaccinia virus). But surprisingly, all modern vaccine strains are composed solely of vaccinia virus,[1,14] which raises the questions of when and where the swap between cowpox and vaccinia virus occurred, if indeed it ever happened. This question is the first mystery in Jenner's story.

Further questions regarding the identity of the material used for vaccination were not answered in Jenner's original manuscript.[5] In addition to the case of Sarah Nelmes, Jenner described seven other cases in which lesions from horses with so-called horse-pox were the source of the vaccine lymph. In his paper, Jenner concludes about cow-pox that "the disease makes its progress from the horse to the nipple of the cow, and from the cow to the human subject."[5] In short, Jenner believed that to be a good source of vaccine, cow-pox should first originate as a disease in horses.

In the early 1800s, Jenner's hypothesis had gained support from part of the medical community after a series of successful vaccination experiments with horse lymph were done by John Loy (1774–1865).[15,16] In 1813, and again in 1817, in the absence of new cases of natural cow-pox, Jenner used lymph from horse-pox lesions as a source of vaccine. The lymph was transferred either to cows and then to people or directly to people, with satisfactory protection power in a process some called equination.[17,18]

At that time, horse-pox was widespread in Europe.[18–21] The disease caused pustular lesions in horses and horse handlers (figure 2), who sometimes transmitted the pathogen to cows during milking activities.[18,21,22] Thus, a second mystery involves the development of Jenner's vaccine. Did Jenner use horsepox virus instead of cowpox virus? The answer is not clear, because vaccinia virus can also infect horses, which develop pustular umbilicated lesions similar to those caused by horsepox virus.[23–25] Thus, one could speculate that either vaccinia or horsepox viruses could have caused the then so-called horse-pox disease in the 18th and 19th centuries. So the mystery persists, did Jenner use cowpox, horsepox, or vaccinia viruses?

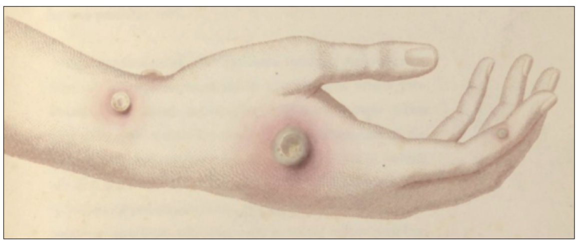

Figure 1: **Cow-pox lesions on the hand of the milker Sarah Nelmes**
Reproduced from Jenner,[5] by permission of Sampson Low.

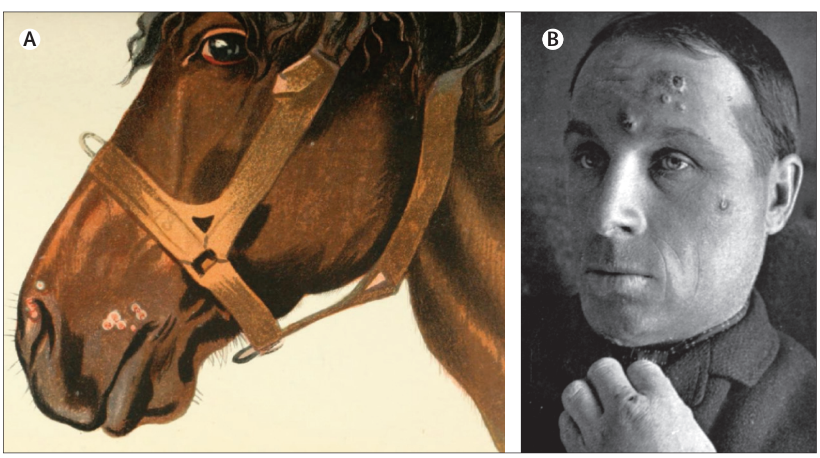

Figure 2: **Horse-pox lesions**
(A) Ancient illustration depicting a horse with horse-pox lesions on the mouth and nose. Reproduced from Crookshank,[18] by permission of P Blackiston's Son. (B) Photograph of a stableman showing horse-pox lesions on the face and hand. The photograph was taken on April 6, 1898. Reproduced from Copeman,[20] by permission of Macmillan.

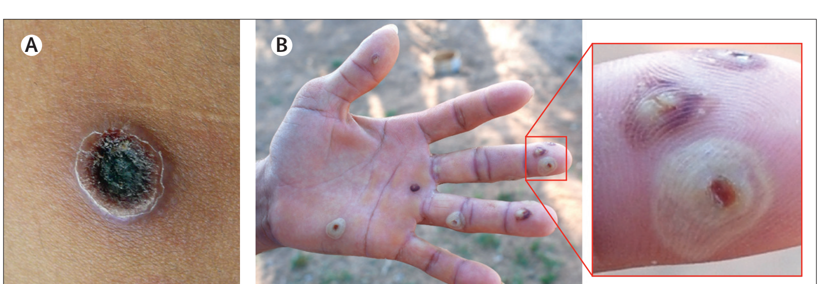

Figure 3: **Zoonotic transmission of cowpox and vaccinia viruses**
(A) Cutaneous lesion on the hand of an individual infected with cowpox virus transmitted by an infected cat. Photo courtesy of Andreas Nitsche, Robert Koch Institute, Berlin, Germany. (B) Cutaneous lesions on the hand of a milker infected with vaccinia virus strain Cantagalo (Cantagalo virus) transmitted by infected cows during milking activities. Photo courtesy of Jociane Quixabeira, Instituto de Defesa Agropecuária do Estado de Mato Grosso, Mato Grosso, Brazil.

The origins of Jenner's vaccine have been intensively debated over the years. British researcher Downie studied the close immunological relationship of variola, cowpox, and vaccinia viruses, which led him to propose in 1951 that vaccinia virus had emerged from cowpox virus in the early years of vaccination.[26] In 1965, Downie strongly opposed Peter Razzell's proposal that vaccinia virus had derived from an attenuated form of variola virus.[27–29] Derrick Baxby (1940–2017) also refuted Razzell's belief and proposed in 1981 that modern 20th century vaccines had derived from horsepox virus.[30–32]

In fact, we currently know that vaccinia virus is more closely related to horsepox virus than to cowpox virus. On the basis of full-genome analysis and recent phylogenetic inferences, horsepox virus is considered an ancestor of the vaccinia lineage.[33–36] Because all modern smallpox vaccine strains consist of vaccinia virus, one would expect that horsepox virus, or even vaccinia virus, would be better candidates than cowpox virus for the original source of Jenner's vaccine. Although an attractive hypothesis, the obscure history of cowpox, horsepox, and vaccinia viruses further adds to the mystery.

Since the mid-20th century, cows have been rarely found sick because of infection by cowpox virus, and only felines, pet rodents, and zoo animals, primarily in Europe, have been affected by the virus in the past decades.[37–40] As for horse-pox, the disease is rare and some believe it is extinct, at least in Europe.[23,24,31,32,41] Only one existing strain of horsepox virus obtained from Mongolian horses in 1976 is currently known.[33] However, vaccinia virus has never been found naturally occurring in animals, except in India, where the strain buffalopox infects buffaloes, and in Brazil, where the strain Cantagalo (Cantagalo virus) infects dairy cows.[2,42–44] In any case, all three orthopoxviruses cause zoonotic infections, and individuals acquire the infection when handling infected animals (figures 2 and 3).[2,22,37,40,45,46]

The good news of a vaccine for smallpox spreads worldwide

Despite initial scepticism about the true power of the vaccine lymph, many physicians in England and continental Europe rapidly adopted the practice of vaccination.[17,47,48] In 1799, George Pearson (1751–1828) founded the Original Vaccine Pock Institute in London, UK, and sent lymph samples to Jean De Carro (1770–1857), a Swiss physician working in Vienna, Austria, in 1800.[49] In the same year, vaccination started in

Germany and France with vaccine material brought by William Woodville (1752–1805) from the Smallpox and Inoculation Hospital in London to a medical committee in Paris.[47,50] From Paris, François Colon (1764–1812) sent the vaccine lymph to Francesc Piguillem (1770–1826) in Spain, who started vaccination in Puigcerdà in 1800 and later in Barcelona.[50,51] A second shipment of the vaccine from Paris to Madrid occurred in 1801.[52,53] Still in 1800, Joseph Marshall and John Walker (1759–1830) took lymph material provided by Jenner to Malta and to the British colonies of Minorca and Gibraltar, heading subsequently to Palermo and Naples in Italy. From Gibraltar, the vaccine also reached Spain.[18,47] The lymph pus was also sent from England to Benjamin Waterhouse (1754–1846) in Boston, MA, USA, in 1800.[17,47,48]

The roles played by Luigi Sacco (1769–1836) from Milan, Italy, and by De Carro in the distribution of the smallpox vaccine in continental Europe and overseas were crucial. Besides exchanging lymph material with each other in the early 1800s they also sent samples to several countries, including England.[20,47,54] The source of the Italian lymph was spontaneous cases of cow-pox in Lombardy, Italy, and its protective power was supported by more than 300 vaccination experiments.[54,55] Successful stocks from equine origin were also obtained by Sacco in 1803 and later in 1812.[20,56]

Early in the 1800s, De Carro started sending lymph material to Turkey, Greece, Prussia, and eastern Europe through British ambassadors. In 1802, De Carro sent the vaccine to Baghdad and Basra in Iraq, and finally to Bombay in India.[47,49] During the trip, the lymph was transferred arm-to-arm, a procedure named Jennerian or humanised vaccination.[18,20] From Bombay, the lymph was distributed to the Portuguese province of Goa, India, and to the islands in the Indian Ocean in 1803. In Australia, vaccination started in 1804.[57]

In Russia, vaccination started in 1801 in the Moscow Foundling Hospital using lymph obtained by the Dowager Empress Maria Feodorovna (1847–1928) from Breslau, Prussia. By 1804, the Free Economic Society, together with several British physicians, had implemented vaccination in several Russian provinces.[47,58,59] In the same year, the vaccine was sent on the arms of slaves from Portugal to Bahia, Brazil, following orders from Felisberto Caldeira Brant Pontes Oliveira e Horta (1772–1842), the Marquis of Barbacena. However, the vaccine potency did not last long.[60,61]

The first worldwide smallpox vaccination campaign took place from 1803 to 1813.[14,51,62–64] The Royal Philanthropic Vaccine Expedition, sponsored by King Charles IV (1748–1819) and coordinated by Francisco Xavier Balmis (1753–1819), left Spain in 1803 towards the Spanish colonies in the Americas and Asia, taking the vaccine lymph that had been transferred arm-to-arm in orphans. In March, 1804, the expedition was divided into two groups when the ships arrived in Venezuela: Balmis headed for the territories of New Spain and then for the Philippines and Macao, reaching Canton (Guangzhou, China) in 1805; and José Salvany (1778–1810) headed for South America.[51,62–64] Years later, Balmis would coordinate another expedition to Mexico (1810–13).[62]

The source of the vaccine used during the Spanish expedition was probably the lymph that had been propagated arm-to-arm in Madrid by Ignacio María Ruiz de Luzuriaga (1763–1822). He received the material from Paris in 1801 and distributed the vaccine throughout Spain.[51–53]

Animal vaccination in Europe: the Beaugency lymph

Different to the humanised vaccine, animal vaccine was propagated by serial passage of vaccine lymph in calves.[1,65] The procedure was implemented by Giuseppe Negri in Naples in 1842, following the principles established by his mentor Gennaro Galbiati (1776–1844) in 1810.[66–68] Animal and humanised vaccination coexisted during the 19th century, but arm-to-arm propagation was progressively banned from several countries, Great Britain being the last to do so in 1898.[1,69] Animal vaccination had important advantages over the humanised vaccine, because the protective power of the lymph was maintained by serial passages, and human-related pathogens, such as those causing syphilis and erysipelas, were not transmitted through the vaccine.[67,68,70]

In 1864, the French physicians Ernest Chambon (1836–1910) and Gustave Lanoix (1837–94) were impressed by the progress on animal vaccination reported by Ferdinando Palasciano (1815–91) at the Medical Conference of Lyon, France. Lanoix went to Naples in the same year to learn about the practice of animal vaccination from Negri.[71] He brought an inoculated cow back to Paris, where together with Chambon, he established the Animal Vaccine Institute in 1864.[20,48,71,72] The virus source was replaced in 1866 by the pustular material obtained from spontaneous cases of cow-pox discovered at two locations in France. The first outbreak occurred at Beaugency in the Loire Valley in April, 1866, and was reported to the Academy of Medicine of Paris. Constantine de Paul, chairman of the Committee on Vaccination of the Academy, certified that the disease was cow-pox and brought inoculated cows back to Paris.[20,72,73] The lymph was passed in cows by Chambon and Lanoix and, in October, 1866, they mixed this material with lymph obtained from a second natural case of cow-pox discovered in Saint-Mandé, in the suburbs of Paris. The mixture was referred to as the Beaugency lymph and was used subsequently by the Animal Vaccine Institute as a seed to produce the vaccine.[20,48,72,74]

The use of animal vaccination established novel quality patterns for lymph production worldwide and new vaccine institutes were established following recommendations by Chambon and Lanoix in Paris. Parameters such as animal age, inoculation method (puncture, scarification,

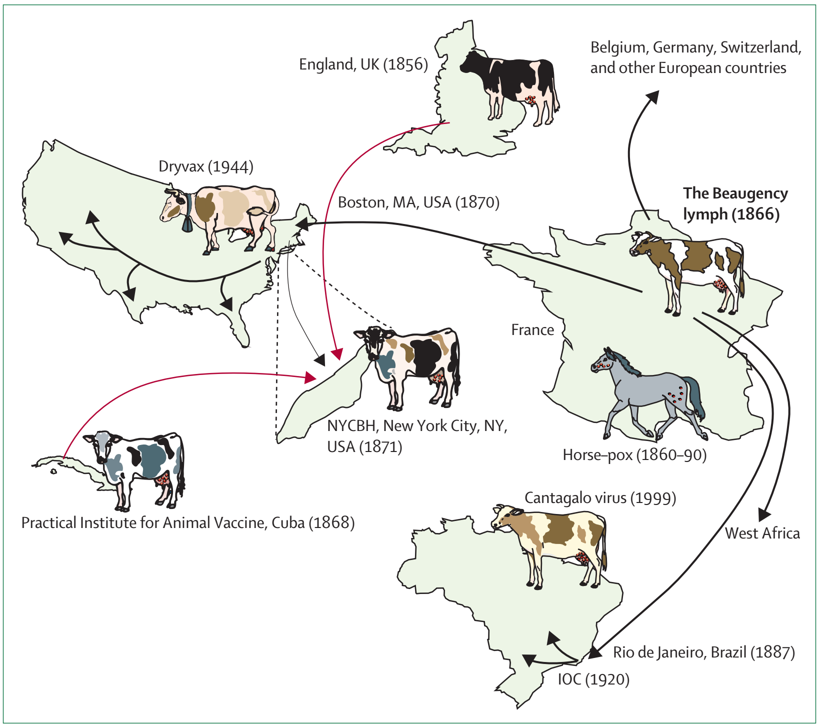

Figure 4: **Schematic worldwide distribution of the Beaugency lymph during the 19th century**
The Beaugency lymph was isolated from natural cases of cow-pox disease in France in 1866 when simultaneous outbreaks of horse-pox were also reported. The lymph was distributed to several European and African countries, and to Brazil and the USA (black arrows). In Brazil, the infectious agent in the lymph found its way into the local wildlife, giving rise to feral vaccinia virus strains (eg, Cantagalo virus) that evolved in parallel with the Brazilian IOC smallpox vaccine. In the USA, the Beaugency lymph was distributed to several cities and was probably used as a major component of the American Dryvax vaccine. By contrast, NYCBH probably used different strains derived mostly from England, UK, and Cuba (red arrows) for vaccine production, with a minor contribution of the Beaugency lymph. Different patterns of cows symbolise the diversity of smallpox vaccine strains that were exchanged and mixed during animal vaccine production in the 19th century. IOC=Oswaldo Cruz Institute. NYCBH=The New York City Board of Health.

or incision), and disinfection were carefully followed.[17,74–76] Germany, however, stood out: vaccination was started in Berlin in 1865 by Eugen Raymund Pissin (about 1830–1907), and by 1874 more than 20 state-controlled institutions for calf-lymph vaccine had been established. Germany had the best smallpox control rates because of compulsory revaccination imposed by the 1874 Revaccination Law, which was then adopted by other countries.[17,77] State control over animal vaccine production was a distinctive feature in European countries, whereas in the USA the vaccine was mostly produced in private animal farms.[76]

In 1869, Henri Blanc (1831–1911) introduced animal vaccination in Bombay,[72] and in 1871, the State Institute for Vaccination was established in Madrid.[78] In Japan, Jennerian vaccine arrived late in 1849 and animal vaccine institutes were not created before 1879.[48,79]

Of note, between 1860 and 1890, several spontaneous cases of horse-pox occurred in France,[18,19] while natural cases of cow-pox occurred for decades in French cities such as Eysines, Bordeaux, and Céron.[80] In 1860, vesicle fluid from infected horses in Rieumes, France, was inoculated on cows' teats and on children's arms by E G Lafosse. Umbilicated vesicles and "a very fine vaccinal eruption" appeared with "all the characters of inoculated Cow Pox".[18] In 1863, a stableman who dressed horses with horse-pox in Alfort, France, developed pustular lesions in his fingers and forehead and lymphadenopathy. The pustular fluid was inoculated into a child and a cow, producing typical vaccinal lesions.[18,21] In 1880, outbreaks of horse-pox in France were reported in Bérat, Rieumes, and Labastide-Clermont. During this period, equine lymph was adopted by some French producers such as the Animal Vaccine Station in Bordeaux, as they believed it was the best lymph source.[18,80]

Nevertheless, it is important to keep in mind that vaccinia, cowpox, or horsepox viruses can infect cows, horses, and people, producing similar umbilicated pustules on their skin.[23–25,38,42,46,81] In any case, whatever the identity of the virus causing the cow-pox cases in Beaugency, the fact is that the protective power of the Beaugency lymph was excellent. Consequently, soon after 1866 the Beaugency lymph was distributed to several European countries and overseas (figure 4). Lymph samples were sent to Belgium, where the State Vaccine Institute was established by Evariste Warlomont (1820–91) in 1868.[20,72,82] Blanc brought Belgian and French samples to London and Bombay in 1869 and successfully passaged both samples in cows.[72] Germany, Sweden, Switzerland, the Netherlands, and the French colonies in west Africa also received the Beaugency stock.[20,70,75,83] In Senegal, the production of smallpox vaccine started in 1895.[84]

The Beaugency lymph reaches the USA

In 1870, Henry Martin (1824–84) of Boston received the Beaugency lymph from de Paul of Paris to start a private animal vaccination farm in the USA.[19,65,73] As in France, excellent protective power was obtained, and the Beaugency lymph was distributed to several American cities.[65,75,76] The first physician to receive the lymph was the director of the New York Dispensary, Frank Foster (1841–1911),[73] who started producing the vaccine consistently in 1871 (figure 4).[85] In the following years, the profitable business of bovine vaccine farms spread rapidly throughout the country.[19,65,76] Animal vaccines were advertised in newspapers and sold by mail in ivory points or quill slips.[86–91]

The northeastern coast of the USA housed the most important animal vaccine producers at the time.[92] The Lancaster County Vaccine Farm in Marietta, PA, was the largest vaccine facility in the USA, with 500 heifers. The business was established in 1882 by H M Alexander (1851–1903), who used the Beaugency lymph obtained from Martin.[93] The company was sold in 1917 to Gilliland Laboratories, which was subsequently incorporated into Wyeth Laboratories in 1943.[94–96] Of note, John Wyeth (1834–1907) and Frank Wyeth (1836–1913), who later founded the Wyeth Laboratories, also had their own animal vaccine farm in Chester County, PA. Their vaccine seed was provided by the Belgian State Vaccine Institute in 1885 to Charles Eucharist de Medicis Sajous (1852–1929) from the Jefferson Medical College, Philadelphia, PA.[97]

By the end of the 19th century, most animal vaccine producers in the country used only the Beaugency lymph as the virus seed, with some exceptions.[65,75,76,92] For example, Francis Martin (1858–1915), son of Henry Martin, used three strains in 1885: the Beaugency lymph, the Belgian Esneux strain, and the Cohasset strain, obtained from a spontaneous case of cow-pox that possibly represented an escape of Beaugency virus from neighbouring farms.[98] Animals were inoculated with the three strains in different body parts as a doubtful attempt to keep the strains pure.

The New York City Board of Health (NYCBH) was the first municipal department in the USA to produce animal vaccine, but the seed strain was not the Beaugency lymph. NYCBH started vaccine production modestly in 1871 by inoculating a few cows with vaccine lymph obtained from the Practical Institute for Animal Vaccine of Cuba and Puerto Rico.[99] The Cuban Institute was founded in 1868 by Vicente Luis Ferrer (1823–83), who had been trained in Europe and had returned to Cuba from Cádiz, Spain, with two calves inoculated with the vaccine virus, supposedly provided by Negri from Naples.[100–102]

In addition to the Cuban lymph, NYCBH also inoculated cows with mixed lymphs, specifically 12 scarification points of the Cuban lymph and two points of the Beaugency stock provided by Foster of the New York Dispensary. Although propagation in calves was satisfactory, humanised vaccine was preferentially used in New York City, NY, at that time.[99] To vaccinate arm-to-arm, NYCBH used a different vaccine strain provided by Jonas Powell Loines (1821–73) of the Eastern Dispensary, who had originally obtained the virus stock from England in 1856 (figure 4).[103,104]

By 1874, humanised and animal vaccines were used indiscriminately, although NYCBH clearly preferred the arm-to-arm vaccine. Nevertheless, objections to humanised vaccination were growing among parents because of so-called blood poisoning after the procedure.[103] Thus, over the subsequent years, NYCBH invested efforts to increase production of the animal vaccine. Farms in Clifton and Paterson, NJ, were used for propagation of the animal lymph. After 1884, the vaccine was mostly produced by a vaccine farm located in the back of the Health Department on Mott Street, in New York City.[105]

The Beaugency lymph reaches Brazil

Throughout the 19th century, there had been several unsuccessful attempts to establish continuous production of smallpox vaccine in Brazil. In 1887, Pedro Affonso de Carvalho Franco (1845–1920)—Baron de Pedro Affonso, physician and director of the Holy House of Mercy in Rio de Janeiro, Brazil—imported the Beaugency lymph from the Animal Vaccine Institute of Paris (Espmark JA to Henderson DA, Sanofi Pasteur archives, March 31, 1969, personal communication). The inoculation of calves with the lymph was a success, and the production of animal vaccine was finally established in the country.[60,106]

In the following years, the successful propagation of the animal vaccine in Rio de Janeiro led Affonso and the Brazilian Government to instigate the establishment of vaccine production centres in other regions of the country. The task was undertaken by Henrique Dodsworth (1865–1916), who visited several provinces in the northeast and took calves inoculated with the Beaugency lymph to São Paulo, aiming to popularise the procedure.[107]

In 1894, the production of animal vaccine was transferred to the Municipal Vaccine Institute of Rio de Janeiro, a private–public enterprise under the supervision of Affonso.[108] In 1920, during the restructuring of the health services in Brazil, the Municipal Vaccine Institute was incorporated into the Oswaldo Cruz Institute (IOC), a federal institution located in Rio de Janeiro (figure 4). The production of smallpox vaccine in calves achieved distinctive status with the use of new technologies, and large-scale production lasted until the late 1970s, when it was finally discontinued.[60,106]

During the smallpox eradication campaign in the 1960s and 1970s, Brazil had a vaccine production centre in each of the four cities: Rio de Janeiro, São Paulo, Porto Alegre, and Recife. However, the IOC in Rio de Janeiro was the major producer and distributor of the smallpox vaccine for the eradication programme across Brazil.[109,110] No report of any kind exists indicating that seed strains other than the one derived from the Beaugency lymph were used by IOC for vaccine production in calves. Thus, the vaccine ampoules produced by IOC until the late 1970s possibly contained virus directly derived from the Beaugency lymph. The other centres used different vaccine strains.[1,110]

In 1999, our research group began a study that compared the IOC vaccine with Cantagalo virus, a strain of vaccinia virus that had been recently isolated from spontaneous cases of pustular disease in dairy cattle in the state of Rio de Janeiro. Our hypothesis was that Cantagalo virus was phylogenetically related to the IOC vaccine, which is currently termed vaccinia virus strain IOC.[2,42] In fact, a recent and detailed study based on full-genome sequences supported this relationship and showed that strain IOC and Cantagalo virus share a most recent common ancestor.[34] We believe that the vaccine virus escaped to nature sometime after its arrival in Brazil in 1887. The transportation of inoculated cows from Rio de Janeiro to other states in the late 1890s might have increased the probability of virus escape.[107] Additionally, there are reports from the mid-1900s of cases of cow-pox in farms in southeastern Brazil where milkers had been vaccinated against smallpox about 1 week before the outbreaks, suggesting that transmission might have occurred from farmers to cows.[2,106,111]

Extant smallpox vaccines and the evolutionary relationship

The 19th century was a time of worldwide exchange and mixing of smallpox vaccine strains and of virus passage through different hosts. These events provided genuine experimental conditions that favoured the evolution of vaccinia virus into pools of highly diverse quasispecies.[17,20,65] This genetic heterogeneity is particularly evident in all existing vaccinia virus strains that have been used as smallpox vaccine. Sequencing and structural analysis of full genomes from isolated viral clones are important strategies that have been used to investigate the genotypic diversity and evolutionary relationships of vaccinia strains.[34,35,112–114]

On the basis of full-genome analysis, the existing vaccinia strains are divided into three main phylogenetic clusters (figure 5). The Eurasian cluster includes different strains used in Europe and Asia such as the British strain Lister, the Chinese strain Tian-tan, and the Russian strain Tashkent.[34,35,112–114] The South American cluster includes clones of the IOC vaccine, the Brazilian field strains of vaccinia represented by Cantagalo and Serro-2 viruses,[42,115] and, surprisingly, horsepox virus (figure 5).[34]

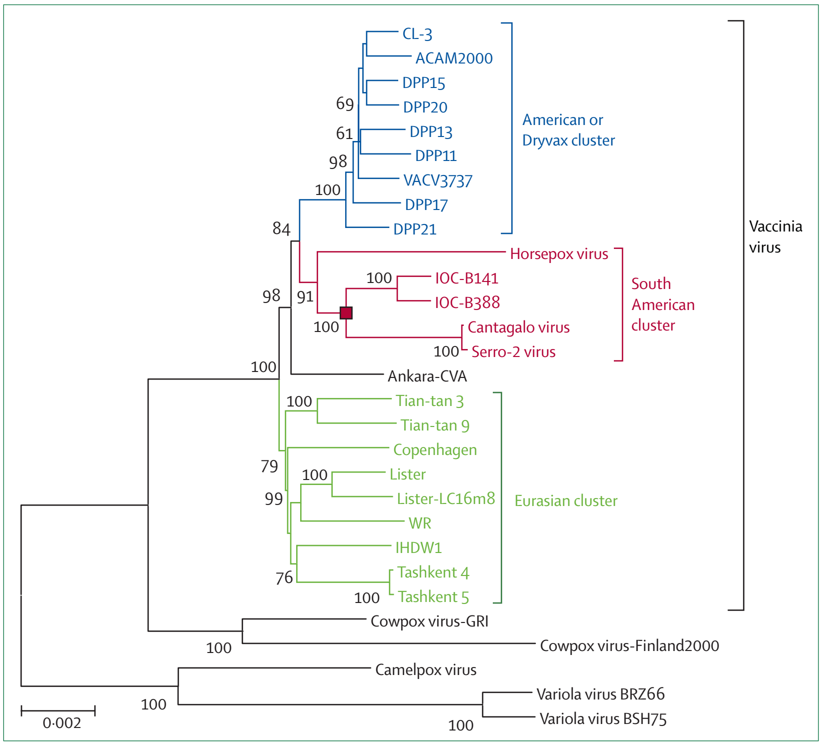

Figure 5: **Vaccinia virus phylogeny**
A multi-alignment of the conserved region (approximately 95 000 nucleotides) of 29 types of orthopoxvirus genomes was used to construct a neighbour-joining tree with use of the Kimura 2-parameter substitution model with 1000 bootstrap replicates. The red square represents the most common recent ancestor of IOC vaccines and the Brazilian field strains of vaccinia viruses. The scale bar indicates the number of substitutions per site.

Horsepox virus is more closely related to IOC than to Cantagalo virus, suggesting that the most recent common ancestor of IOC and Cantagalo virus was probably an ancient vaccine strain related to horsepox virus. The fact that the Beaugency lymph is the only known ancestor strain introduced in Brazil since 1887 for production of animal vaccine by the IOC indicates that Beaugency strain and horsepox virus are closely related viruses. This fact supports the hypothesis that the Beaugency lymph was in fact either a vaccinia virus or a horsepox-related virus, and not cowpox virus as previously thought. Notably, all phylogenetic studies are based on a single existing sample of horsepox virus, and it is unknown whether this 1976 virus represents a true autochthonous strain or that it might be a vaccine escapee.[33,112] In any case, its genome has certainly evolved and diverged from ancient 18th and 19th century horsepox viruses. Therefore, further sequencing studies of old smallpox vaccines might support the hypothesis of a Beaugency–horsepox correlation.

The third vaccinia cluster is the American or Dryvax cluster that groups all viral clones isolated from the American vaccine Dryvax, including ACAM2000, the current licensed smallpox vaccine in the USA.[35,112,113] Phylogenetic inference places this cluster as a sister group to the South American cluster (figure 5).[34] Depending on the number or clone of Dryvax strain used in the inference, the mapping of horsepox virus fluctuates, branching off the base of the American cluster.[34]

Dryvax is a calf-based freeze-dried vaccine supposedly originating from the NYCBH strain.[1,104] The WR strain and IHD strain, which apparently are also derived from the NYCBH strain,[1,104] group consistently within the Eurasian cluster.[34,35,112–114] Despite support in the literature for an exclusive NYCBH origin of the Dryvax strain, historical evidence suggests otherwise. In fact, it has been reported that Wyeth Laboratories obtained a sample of the vaccine strain from NYCBH in the early 1900s.[1,116] However, the Wyeth Laboratories were licensed to manufacture Dryvax in 1944,[117] when production started in its acquired vaccine plant in Marietta, where H M Alexander had propagated the Beaugency lymph.[94,95,117] Moreover, the Wyeth brothers also had a vaccination farm in Pennsylvania and used the lymph obtained from the Belgian Government.[97] Hence, it seems plausible to suggest that the Beaugency lymph was used for manufacturing Dryvax (figure 4).

The use of the NYCBH strain in the initial Dryvax vaccine cannot be ruled out but it is possible that NYCBH was not a pure strain. After 1871, different vaccine strains were in fact used to produce the NYCBH smallpox vaccine. Thus, we hypothesise that the Dryvax vaccine consists of mixed vaccine strains with a strong component of the Beaugency lymph. This hypothesis is supported by the phylogenetic data (figure 5), showing the relatedness of the American and South American

Search strategy and selection criteria

I searched PubMed, Google Scholar, the archives of the Spanish Royal Academy of Medicine, the archives of Columbia University Libraries, and the Internet Archive (particularly the archives of the American libraries) from Jan 1, 1798, to Jan 31, 2017, for articles, books, and pamphlets. I used the search terms "smallpox vaccine", "horse-pox", "cow-pox", "vaccinia", and "Beaugency lymph". References in English, Portuguese, Spanish, Italian, and French were inspected. Relevant references cited in these articles and books were also examined. To investigate the origins of the Brazilian vaccine strain used by the Oswaldo Cruz Institute, a physical search of the Robert Wilson smallpox eradication files at Sanofi Pasteur (Connaught campus, Toronto, ON, Canada) was done. To investigate announcements of vaccine sale, animal vaccine farms, and business transactions involving vaccine farms and pharmaceutical companies, I searched Newspapers.com for historical American newspapers in English from Jan 1, 1866, and Dec 31, 1950, using the keywords "Wyeth", "Beaugency", "animal vaccine", "bovine lymph", "Gilliland", "Marietta plant", and "New York City Board of Health". Studies involving the genotypic diversity of vaccinia virus were selected by searching PubMed with no language restrictions from Jan 1, 1980, to Jan 31, 2017, with the search terms "Brazilian vaccinia", "Dryvax", and "vaccinia virus evolution".

clusters, and it explains why WR and IHD strains do not group with the American cluster (but with the Eurasian cluster instead), and also supports the idea that WR and IHD strains originated primarily from the NYCBH strain.[104] Again, caution is needed when analysing phylogenetic inferences of vaccinia viruses. Virus mixing in the 19th century possibly favoured multiple recombination events between vaccinia genomes, leading to the introduction of interesting structural features in the genome ends.[35] These features contribute to the understanding of the vaccinia lineage, but might introduce inconsistencies in phylogenetic trees; therefore, vaccinia virus genome ends deserve a separate structural analysis.[34,35,118]

Conclusion

The history and evolutionary relationships of the different smallpox vaccine strains remain obscure, because most strains and clonal variants have not yet been completely sequenced. Historical and scientific evidence suggests that the Beaugency lymph and horsepox virus made a strong contribution to the genetic makeup of the existing vaccine strains, particularly those from continental Europe, USA, and Brazil. Evidence also suggests that the Beaugency lymph was probably a vaccinia virus strain or a close ancestor related to horsepox virus, and not a cowpox strain. Thus, it is reasonable to believe not only that cowpox virus was not used as a smallpox vaccine in the 19th century, but that it might have never been used at all. Assumptions about the origins of the Beaugency lymph also indicate that vaccinia virus was once a circulating virus in animals in Europe in the 19th century, but later it disappeared and now has no natural hosts, except in Brazil, India, and perhaps in Egypt, Pakistan, Bangladesh, and Nepal. Many questions still persist, and analysis of full-genome sequences of ancient vaccine strains would help unravel this fascinating medical mystery.

For the **archives of the Spanish Royal Academy of Medicine** see http://www.ranm.es

For the **archives of Columbia University Libraries** see https://clio.columbia.edu/

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I am the sole author and contributor to this manuscript.

Declaration of interests
I declare no competing interests.

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