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Review

Equination (inoculation of horsepox): An early alternative to vaccination (inoculation of cowpox) and the potential role of horsepox virus in the origin of the smallpox vaccine

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ABSTRACT

For almost 150 years after Edward Jenner had published the "Inquiry" in 1798, it was generally assumed that the cowpox virus was the vaccine against smallpox. It was not until 1939 when it was shown that vaccinia, the smallpox vaccine virus, was serologically related but different from the cowpox virus. In the absence of a known natural host, vaccinia has been considered to be a laboratory virus that may have originated from mutational or recombinational events involving cowpox virus, variola viruses or some unknown ancestral Orthopoxvirus. A favorite candidate for a vaccinia ancestor has been the horsepox virus. Edward Jenner himself suspected that cowpox derived from horsepox and he also believed that "matter" obtained from either disease could be used as preventative of smallpox. During the 19th century, inoculation with cowpox (vaccination) was used in Europe alongside with inoculation with horsepox (equination) to prevent smallpox. Vaccine-manufacturing practices during the 19th century may have resulted in the use of virus mixtures, leading to different genetic modifications that resulted in present-day vaccinia strains. Horsepox, a disease previously reported only in Europe, has been disappearing on that continent since the beginning of the 20th century and now seems to have become extinct, although the virus perhaps remains circulating in an unknown reservoir. Genomic sequencing of a horsepox virus isolated in Mongolia in 1976 indicated that, while closely related to vaccinia, this horsepox virus contained additional, potentially ancestral sequences absent in vaccinia. Recent genetic analyses of extant vaccinia viruses have revealed that some strains contain ancestral horsepox virus genes or are phylogenetically related to horsepox virus. We have recently reported that a commercially produced smallpox vaccine, manufactured in the United States in 1902, is genetically highly similar to horsepox virus, providing a missing link in this 200-year-old mystery.

Contents

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1. Introduction

Vaccination was largely responsible for the eradication of smallpox declared by the World Health Organization in 1980 [1]. Most people are familiar with the work of Edward Jenner, who in 1798 published his famous work, often simply referred to as the "Inquiry", reporting that the inoculation of cowpox protected against smallpox [2]. Jenner called this new procedure "vaccine inoculation", to make reference to the cow (Lat. *vacca*), the proposed origin of the preventative against smallpox. Richard Dunning, a surgeon in Plymouth, coined the term vaccination in 1803 [3]. With the development of the germ theory of disease in the second half of the 19th century, a large number of specific microorganisms were identified as the etiological agents of different human and animal diseases. At the 7th International Congress of Medicine in London in 1881, to honor Jenner, Louis Pasteur proposed to generalize the term "vaccination" to include all protective immunization procedures against any infectious diseases [4,5].

A little-known fact is that Edward Jenner himself considered that the preventative against smallpox which is present in cowpox lesions, in fact derived from a disease of horses known as "grease" (horsepox). Early in the 19th century, European physicians conducted experiments that seemed to confirm the hypothesis that horsepox could protect against smallpox. Because of the relative rarity of cases of spontaneous cowpox, matter obtained from either cowpox or horsepox was interchangeably used in the protection against smallpox. Congruent with the use of the word vaccination, the inoculation from the horse (Lat. *equus*) was referred to as "equination" [6–8].

For much of the 19th century the true nature of the preventative against smallpox was discussed among the medical community, although it was usually assumed to be derived from cowpox [9]. It is important to appreciate that although the broader concept of transmissible agents of diseases was generally accepted by 19th century science, the medical community had to wait until the end of the century to have a clearer understanding of the role of microorganisms in the causation of disease. Thus, smallpox vaccination was developed and used in the 19th century based on enlightened empiricism. Viruses were only identified at the very end of the 19th century, based mainly on the criterion that their small size allowed them to pass through filters known to retain the smallest bacteria (filterable viruses).

The science of virology made considerable advances during the first half of the 20th century, including the ability to multiply viruses in embryonated eggs and tissue culture, thus allowing for a more detailed characterization of their biological, biochemical and immunological properties [10]. In 1939 Allan Watt Downie, a Professor of Bacteriology at the University of Liverpool, using serological techniques, demonstrated that the contemporary virus used for vaccination against smallpox, now referred to as vaccinia, was different from cowpox virus [11,12], reopening the scientific discussion about the true origin of vaccinia [13]. Since a natural animal host is not known, vaccinia is frequently referred to as a laboratory virus that could have originated from a still unidentified animal Orthopoxvirus ancestor. Based on the historical record, several investigators, especially the recently deceased Derrick Baxby (1940–2017), then a Lecturer in Medical Microbiology at the University of Liverpool, proposed that a presumed horsepox virus could be the long-sought ancestor of vaccinia [14–18]. Horsepox is a very rare disease that may have become extinct [19]. Fortunately, samples from a 1976 case of horsepox from Mongolia were used for genome sequencing, revealing that the horsepox virus is genetically related to vaccinia and might even be one of its ancestors [16]. Subsequently, other investigators have reported that contemporary vaccinia strains may in fact represent viruses derived from complex recombinational events between different strains of vaccinia that may have included a horsepox virus ancestor [20–22]. We recently reported that a smallpox vaccine commercially produced in the United States in 1902 is closely related to horsepox virus, confirming the hypothesis that at least some of the early smallpox vaccines were based on horsepox virus [23].

Here we review the historical data on the early use of horsepox to immunize against smallpox (equination), as well as relevant scientific information regarding vaccinia virus and its two most likely ancestors, cowpox and horsepox viruses, in an attempt to throw light on a 200-year-old mystery, the origin of vaccinia.

2. The historical record on equination

2.1. Jenner used both vaccination and equination to protect against smallpox

The "Inquiry", the most important work of Edward Jenner, was published in 1798 under the title of "An Inquiry into the causes and effects of the variolæ vaccinæ, a disease discovered in some of the western counties of England, particularly Gloucestershire, and known by the name of the cow pox" [2]. After documenting a number of people in which previous infection with cowpox protected against smallpox (or against inoculation of smallpox, an early prophylactic procedure also known as variolation), on 14 May 1796, Jenner inoculated an eight-year-old boy named James Phipps with matter obtained from a cowpox pustule on the hand of Sarah Nelmes, a dairy maid, who had been directly infected from a cow. That was the first and best-known experimental inoculation of cowpox done by Jenner. To confirm that the child was protected against smallpox, he was variolated six weeks later without showing any evidence of infection, providing the first experimental evidence that cowpox elicits protection against smallpox. At a time when the nature and properties of viruses were not known, Jenner had the intuition that the cause of cowpox was related to that of smallpox, and for this reason he invented the term of "variolæ vaccinæ", or "smallpox of the cow", to refer to cowpox.

Other authors have analyzed the strengths and weaknesses of the arguments advanced by Jenner to promote the use of cowpox as a preventative of smallpox [3,13,14,24–30] and those arguments will not be repeated here. We will focus instead on those aspects of the Inquiry that specifically relate to equination.

Jenner considered the question of the origin of "spontaneous" cases of cowpox and he made the observation that such cases were frequently observed in farms where the same workers took care of diseased horses and milked cows. He suggested that these farm workers were responsible for transferring the putative agent of cowpox from horses to cows. In fact, on the second page of the Inquiry [2], Jenner made the following comments: "*There is a disease to which the Horse, from his state of domestication, is frequently*

subject. The Farriers have termed it the Grease. It is an inflammation and swelling in the heel, from which issues "matter" possessing properties of a very peculiar kind, which seems capable of generating a disease in the Human Body (after it has undergone the modification "which" I shall presently speak of), which bears so strong a resemblance to the Small Pox, that I think it highly probable it may be the source of that disease. In this Dairy Country a great number of Cows are kept, and the office of milking is performed indiscriminately by Men and Maid Servants. One of the former having been appointed to apply dressings to the heels of a Horse affected with the Grease, and not paying due attention to cleanliness, incautiously bears his part in milking the Cows, with some particles of the infectious matter adhering to his fingers. When this is the case, "it commonly" happens that a disease is communicated to the Cows, and from the Cows to the Dairy-maids, which spreads through the farm until most of the cattle and domestics feel its unpleasant consequences. This disease has obtained the name of the Cow Pox."

The Inquiry reported 23 "cases", either observations or experimental inoculations, which Jenner used to provide objective evidence for his hypothesis [2]. Sarah Nelmes was case XVI and James Phipps (although his name was not given in the publication) was case XVII. It is little known, however, that at least five of the 23 reported cases (XIII, XIV, XV, XVIII and XX) involved matter that Jenner believed was directly or indirectly derived from horses. Case XV acquired the disease directly from a horse, although twenty years later he contracted smallpox, and Jenner speculated that the protection afforded by matter derived from horses was incomplete, because the morbid material from the horse needs to first infect "*the nipple of the cow, and passed through that medium to the human subject*." Case XX was William Summer, a child who was inoculated with matter taken from the nipples of one infected cow from a farm in which cowpox broke out, presumably through contamination from an infected horse. The case of William Summers is made special by the fact that he was the source of a chain of human-to-human transmission, which was one of the most remarkable contributions of the Inquiry, and the observation that initially allowed the vaccine to be extensively used throughout the United Kingdom, Europe and the rest of the world.

The publication of the Inquiry was received with a mixture of expectation, skepticism and criticism. On the one hand, vaccination could represent a safer alternative to variolation, a procedure that carried considerable risks for the individual (up to a 2% chance of developing severe smallpox and dying) and for the population (by starting smallpox epidemics) [24]. On the other hand, some people argued that there were known cases in which previous infections by cowpox did not confer protection against smallpox. In this regard, Jenner went to extraordinary lengths to describe that only the "true cowpox" was an effective preventative against smallpox and no other disease of the cow that he referred to as "spurious cowpox". Jenner was right in making this distinction, because today we know of other diseases that could be confused with cowpox, such as bovine mamillitis caused by bovine herpesvirus 2, milker's nodule caused by a poxvirus of the genus Parapoxvirus, or warts [31].

It has been said that after publishing the Inquiry, Jenner abandoned the hypothesis of the horse origin of the preventative against smallpox. In fact, in his subsequent works about the smallpox vaccine, published between 1779 and 1801 [32–34], Jenner did not make any explicit reference to the potential horse origin of cowpox. The reality is that although in public Jenner downplayed a potential role of grease, probably to avoid unnecessary distractions from his main findings, in private he continued favoring the possibility that horse grease could also be a preventative of smallpox.

Experimental confirmation of a potential horse origin of the preventative of smallpox was complicated by Jenner' confusing grease (dermatitis verrucosa) with horsepox (variola equina), which may have hampered a more systematical evaluation of the potential role of horsepox, or its wider use as a preventative for smallpox [35].

In a 1797 letter to Jean de Carro (a Geneva-born physician practicing in Vienna, who played an important role in introducing the vaccine in Europe, the Near East and India) [36–38], Jenner expanded on what he believed was the mechanism by which the protective matter was transmitted from horses: "*If the Cowpox be unknown in the Country in which you dwell, I should presume that Men Servants, who are employ'd among Horses, are not employ'd in milking cows. In Ireland, & in Scotland, where the Men Servants do not milk, the disease is also unknown. It is unlucky (if I am right in my opinion of the origin of the disease) that we cannot communicate it in a direct way from the Horse to the Cow*".

Jenner went back to his hypothesis of the horse origin of the vaccine in an 1804 letter to the Geneva physician Alexander Marcet: "*There was a circumstance in my first publication which escaped the attention of almost all my Readers, perhaps even you, and that is my second Plate, which represents a Pustule on the arm from a virus derived from the Horse & not the Cow* (from case XVIII, John Baker)" [39].

Jenner and other British physicians were commonly using equine matter for inoculation without specifically mentioning it, probably because vaccinators were comfortable with the concept that all the vaccines against smallpox were one and the same, regardless of their animal origin.

In a series of letters written in 1813 to James Moore, Director of the National Vaccine Establishment in London, Jenner described his use of equination [9]. In June 1813 he indicated that a Mr. Melon, a surgeon of repute in Litchfield, "*has sent me some of his equine virus, which I have been using from arm to arm for these two months past, without observing the smallest deviation in the progress and appearance of the pustules from those produced by the vaccine*" [39]. In a follow-up letter to Moore, dated August 1813, Jenner stated that "*I have been constantly equinating for some months, and perceived not the smallest difference between the pustules thus produced and the vaccine. Both are alike, because they came from the same source.*"

According to Crookshank [9,40], a distinguished British bacteriologist who 90 years after the publication of the Inquiry became one of the severest critics of Jenner, Jenner had abandoned vaccination for Equination by 1817. In effect, in May 1817 Jenner provided a stock of equine lymph to the National Vaccine Establishment, which according to Baron was widely distributed [39].

2.2. Early independent evidence of the protective role of equination

The first independent evidence in support of a horsepox role in the prevention of smallpox was published in 1801 by John Glover Loy, a Yorkshire physician [41]. Loy described several cases of people who became infected while treating horses suffering from grease or of individuals inoculated with horse matter that had been passaged in cows. Loy reported that the lesions produced had exactly the appearance of genuine cowpox, and in many cases the individuals were shown to be protected from variolation. A most important observation made by Loy was that "*two kinds of Grease exist, differing from each other in the power of giving disease to the human or brute animal*", an observation that mirrors Jenner's description of "true" and of "spurious" cowpox, which could explain the lack of success of some earlier investigators who attempted to equinate. The work of Loy was not in total agreement with Jenner's original hypothesis which required that the horse matter was first modified by a passage in the cow before it could fully function as a preventative of smallpox. Nevertheless, Jenner considered that Loy's work "*decisively proves (his) early assertions*" about the horse origin of vaccinia, although Jenner never again adopted the horsepox theory in his publications [14].

Additional confirmation of the horsepox hypothesis was provided by Luigi Sacco, an Italian physician responsible for the early introduction and widespread use of vaccination in Northern Italy [42]. In a letter from Sacco to Jenner, dated 25 March 1803, he explains that after reading the book by John Loy, he was encouraged to continue his own experiments to try to obtain the vaccine from horses with grease. Sacco described two individuals who became infected from horses with grease (referred to as "Giavardo" in Sacco's publications in Italian), from whom he inoculated children and cows. Matter from the inoculated children was used to vaccinate other children who, subsequently, became resistant to variolation. With that experience, Sacco became convinced that grease was the origin of the smallpox preventative and that a passage in cows was not necessary to confer the ability to protect against smallpox. In the same letter, Sacco suggested to Jenner that, perhaps, he should consider changing the name of vaccine for that of equine [6,39].

2.3. Other historical observations

Although the use of cowpox as a vaccine against smallpox was the method of choice during the 19th century, additional efforts were made by some vaccinators to document that at least certain forms of grease, those compatible with horsepox, could also be used to protect against smallpox.

In a letter from the Geneva physician and vaccinator Jean de Carro to Jenner, dated 21 June 1803, he describes the experiences of a Monsieur La Font, a French physician established in Salonica in Macedonia (in present-day Greece), who succeeded in inoculating two children with matter obtained from a horse suffering of grease (that he called "Javart"), although no information was provided regarding its protective value against smallpox [39].

Luigi Sacco sent his horse-grease matter to Jean De Carro, in Vienna, who used it freely and gave of it to others [43]. Many years later, in 1825, De Carro, who frequently added to his signature "vaccinator et equinator", wrote a letter to his Edinburgh Professor of Anatomy, Alexander Monro (the third), indicating that "*the matter used at Vienna from 1799 to 1825 was partly British vaccine, and partly originated from the grease of a horse at Milan, without the intervention of the cow. The effect was so similar in every respect that they were soon mixed; that is to say, after several generations, and, in the hands of innumerable practitioners, it was impossible to distinguish what was vaccine and what was equine*" [43,44].

Additional reports came from Paris where in 1812 a coachman became infected while dressing a horse affected with the grease, from whom two children were vaccinated and from them a vaccine stock was prepared to vaccinate many others [39,45].

Derrick Baxby mentioned that in the early 19th century many equine strains were introduced in Great Britain and were used by Jenner, but unfortunately no detailed accounts of their development were published [14].

Edgar Crookshank [9] was unable to obtain any data on horsepox cases in Great Britain but he was able to document several examples in France, including a large outbreak of grease at Rieumes, near Toulouse, in 1860, where matter from affected horses was successfully used to inoculate a cow and more than 200 children, inducing typical vaccine lesions that protected against a challenge with "ordinary vaccine virus" [46]. Another case of horsepox was documented in 1863 in Alfort, now a suburb of Paris, where a student was contaminated from a horse and the matter subsequently used to vaccinate a child. Another outbreak of horsepox occurred in Toulouse in 1880, from which a heifer and several children were vaccinated.

In 1889 Crookshank concluded that in Great Britain "it is more than probable that some of the Jenner's stocks of equine lymph are still in use; but equination is not wittingly practiced, for it is commonly supposed that all the lymph employed for the purpose of vaccination has been derived from Cow Pox. In France, on the other hand, it is extensively employed" [9].

In 1887, Pierre-Victor Galtier, Professor at the Veterinary School in Lyon, France, reported several cases of horsepox in the town of Saint-Jean-de-Nay, in the French department of Haute-Loire, from where matter was obtained to inoculate eventually hundreds of children [47]. It is interesting that some French vaccinators argued that the horsepox-originated vaccine could be passed from one calf to another for at least eleven generations without losing potency, while the cowpox material usually lost potency after only four passages [48].

Many different smallpox vaccine stocks from Europe were introduced to the United States during the 19th century. It is commonly assumed that most vaccines used in the United States by the end of the 19th century derived from the so called "animal vaccine" (which has never been passaged in humans) introduced in 1870 by the British-born Boston physician Henry Austin Martin, reportedly originating from a spontaneous case of cowpox that occurred in Beaugency, France, in 1866 [49,50]. However, Martin reported that he had also used lymph "*from a heifer inoculated from a spontaneous case of "horse-pox", the fully-proved origin of the disease as occurring in the cow*" and that he "*intend(ed) to continue the propagation of the two "stocks" mentioned above*" [51]. Since neither cowpox nor horsepox are found in the Americas, it is not clear what the origin of Martin's horsepox virus was.

A well-documented case of intentional equination happened in Holland as late as 1914, when Professor De Jong, from the University of Leyden, observed an epizootic of pustular stomatitis (horsepox), providing matter which was successfully used to immunize nine children [52].

Taken together, it is well documented by historical data that the practice of equination was widely used by multiple physicians in several countries across Europe to protect successfully against smallpox.

3. A tale of three Orthopoxviruses

The development of the smallpox vaccine took place more than 100 years before the modern concepts of virology were established. Nevertheless, Edward Jenner and other smallpox vaccine researchers of the 19th century showed extraordinary capacity to infer complex scientific facts from careful epidemiological and clinical observations, including the identification of the immunological relationships that exist among cowpox, horsepox, vaccinia and variola viruses. Today we know that variola, the agent of smallpox, as well as the cowpox, vaccinia and horsepox viruses are all immunologically related viruses grouped within the Orthopoxvirus genus of the Poxviridae family. Traditionally the diseases they cause, and the associated viruses, were defined and named after the most obvious affected host, such as cowpox for the virus causing pocks in cows, or horsepox for that causing similar pathology in horses [53].

3.1. Cowpox virus, a virus from Eurasian rodents

In Jenner's time, cowpox was described as a disease characterized by the appearance of lesions which are successively papular, vesicular and pustular, normally confined to the teats and udders of the cows, that occurred sporadically or as epizootics [7,54]. It is believed that the disease existed in Europe "from time immemorial", although it was the work of Edward Jenner that attracted attention to this disease.

Although it is generally believed that the occurrence of cowpox was not frequent in Great Britain at the end of the 18th century, in

Gloucestershire alone, the county where Jenner conducted his observations and experiments, there were at least ten cowpox epizootics between 1759 and 1798, suggesting that such epizootics occurred regularly [55]. In 1888, Edgar Crookshank published his investigations of a cowpox outbreak in Wiltshire, a location relatively close to Gloucestershire. He discarded the possibility that the outbreak originated directly from horses and argued that the main mode of dissemination was by mechanical transfer by milkmaids from diseased horses to healthy cows, just as suggested by Jenner almost 100 years earlier [56].

At the turn of the 19th to the 20th century, most human cases of cowpox occurred among dairy farm workers, to the point that cowpox was considered to be an occupational disease [57,58]. In 1977, Baxby reported 12 separate cases of confirmed cowpox occurring in Great Britain between 1965 and 1976, ten of which in humans, and conducted a serological survey among 1076 cows in the same general region where the cowpox cases were reported, finding that only 0.7% of them had Orthopoxvirus antibodies. Baxby failed to identify the source of the human cases, suggesting that small wild animals could play a role as hosts and vectors of cowpox [59]. At the same time, other authors were reporting that domestic cats were the most frequently recognized host of the cowpox virus, raising the possibility that cats represented an intermediate host between hypothetical infected wild rodents and humans [60]. Serological tests conducted among different species of rodents in various Eurasian countries confirmed the hypothesis that different species of wild rodents serve as the reservoir of Orthopoxviruses, including cowpox virus [60–63]. Cases of cowpox in cows have not been reported in the last few decades in Great Britain, perhaps due to changes in animal husbandry practices and the almost universal use of the vaccine for most of the 20th century. In contrast to variola virus that can only infect humans, cowpox virus has the broadest host range of all known Orthopoxviruses [64], and an increasing number of cowpox cases have been described, not only in a variety of species of wild rodents but also in cats, zoo animals and unvaccinated humans in Europe.

Different strains of cowpox virus differ in their *in vitro* characteristics and pathogenicity in mice [65,66], and one can only wonder if those differences had also been important in the 19th century in the voluntary or involuntary selection of the less reactogenic cowpox virus strains for vaccine use.

An additional level of diversity among cowpox viruses has been shown by the phylogenetic analysis of their genomes [67–69]. Those studies indicate that cowpox viruses may not represent one single virus species and that the different isolates in fact can be grouped in at least three to five distinct clades. In this regard, it has been pointed out that, despite the general belief that the smallpox vaccine originated in the United Kingdom, the phylogenetic analysis shows that vaccinia strains map closer to Russian and Finnish isolates of the cowpox virus, than to British isolates [67].

3.2. Horsepox virus, a probably extinct virus

Ever since Jenner suggested in 1798that horse grease was the origin of cowpox, the very existence of this disease has been mired in controversy. This has led some modern authors to indicate that "*Horsepox is a tantalizing disease for a modern virologist who is interested in the history of Jenner's vaccine*" [70].

Nevertheless, grease was known by Jenner's contemporaries. Writing in 1803, Henry Marie Husson, secretary of the Parisian Medical Committee for the Inoculation of the Vaccine described that "*During the wet seasons, or well after long toils, horses are frequently sick of a disease named "the grease" in English and "Eaux-aux-Jambes" in French* (water in the legs). *It is a cutaneous disease, generally chronic, sometimes inflammatory and contagious, never acute, that affects the skin of the extremities of the horse, of the ass and the mule, but rarely of the ox*" [71,72].

In fact, grease heel, also known as dermatitis verrucosa or chronic pastern dermatitis, has been known for centuries [73]. However, it has been argued that Jenner may have confused grease with horsepox, or variolæ equinæ, also known as scratchy heel, even though the two conditions were well known and recognized at that time [35]. The confusion between the two diseases was probably the reason for the early failures to reproduce Jenner's findings related to horsepox immunization, somehow clarified by the work of John Loy in 1801 [41]. Equine pastern dermatitis is not a single disease but a cutaneous reaction of the horse that could be triggered by multiple causes, including genetic and environmental factors, allergic reactions and multiple bacterial infections [74]. By 1863 Henry Marie Bouley and other French veterinarians introduced the English term of horsepox to distinguish this disease from the syndromic equine pastern dermatitis or eaux-aux-jambes [75].

Classical horsepox was considered to occur only in Europe, and it was characterized by pox-like lesions in and around the mouth and the legs of horses [76,77]. Like in the case of cowpox, cases of horsepox were relatively uncommon even in Europe, and the disease began to disappear from that continent at the end of the 19th century. The latest human cases of horsepox directly transmitted from horses were reported from Great Britain early in the 20th century [78,79].

Although horsepox now seems to have become extinct in Europe, severe cases of the disease were reported in the Khentii province of Mongolia in 1976 [16]. However, in the 1980s, horsepox occurred only sporadically in Mongolia, especially in the Domogovi, Dundgovi and Khentii provinces (Pip Beard and Greg Gray, personal communication) [19]. Another potential horsepox epizootic had originally been reported in the Uasin Gishu county of Kenya in 1934, although the causative virus was not genetically characterized [80,81]. Recent efforts to identify contemporary cases of horsepox anywhere in the world have failed [19].

The only horsepox virus genome that has been sequenced is from a 1976 isolate from Mongolia (MNR-76), revealing important information regarding the genetic relationships of cowpox, horsepox and vaccinia viruses [16]. The horsepox virus genome (212,633 base pairs) has an intermediate size between vaccinia (177,923–194,711 base pairs) and cowpox (223,666–228,250 base pairs) viruses, and a phylogenetic analysis of its central conserved region revealed that horsepox virus is closely related to but distinct from vaccinia. The authors concluded that while being closely related to the known vaccinia-like virus, horsepox virus contains additional, potentially ancestral sequences absent in other vaccinia viruses. However, it is interesting to note that the horsepox virus genome sequence has genes that are fragmented in some vaccinia strains, but it also has fragmented sequences that are complete in some other vaccinia strains [16]. This observation suggests that several horsepox virus strains could have served as ancestors of different vaccinia lineages.

We believe that the characterization of additional horsepox virus isolates is needed to have a clearer idea of the phylogenetic relationships of this virus with cowpox virus and their contribution to the origin of the different lineages of vaccinia. Although the clinical identification of new cases of horsepox is an obvious activity to be conducted, we also believe that it is important to conduct serological and virological studies of relevant rodent populations which, as in the case of cowpox, could serve as the virus reservoir in nature [19].

3.3. Vaccinia virus, the contemporary vaccine against smallpox

Vaccinia is the name given to the virus used to manufacture the vaccine used to eradicate smallpox. The name obviously derives from "variolæ vaccinæ", or smallpox of the cow, the name that Jenner invented to refer to cowpox, that for more than one 100 years was believed to be the preventative against smallpox [82]. The cowpox hypothesis was not without controversy and other possibilities were advanced, including the one that the vaccine was an attenuated form of variola [43,83–87]. In 1889 Taylor asked the important question: "*What is vaccinia? Is it cow-pox, or horse-pox, or grease cow-pox, or horse-pox cow-pox, or small-pox cow-pox?*", *concluding* that "*One problem the Royal Commission on Vaccination has to solve is, What is Vaccinia? If they solve this one point only, their time will not be altogether wasted*" [88].

The Italian microbiologist Adelchi Negri showed in 1905that vaccinia passes through bacteriological filters [89,90], and in 1913 vaccinia was grown for the first time in the laboratory [91]. In 1922 vaccinia was purified using high-speed centrifugation [92] and in 1940 DNA was shown to constitute its genetic material [93].

All the above research work was taking place while the field could not agree on the true origin of vaccinia [94]. The discussion was reopened when in 1939 Downie used a number of serological tests (neutralization, agglutination and complement-fixation) to compare two strains of vaccinia with two strains of cowpox virus, reaching the conclusion that the virus causing spontaneous cowpox is not the same as the strains of vaccinia virus examined, although the author could not discard a potential derivation from variola [12]. In the late 1970s and mid-1980s, the true nature of vaccinia began to be addressed by the genetic analysis of the virus genomes. DNA endonuclease restriction site mapping confirmed molecularly that cowpox, vaccinia and variola viruses were closely related but distinct viruses [95,96], observations that were fully confirmed by full genome sequencing [67–69,97,98].

Since no natural animal host is known for the vaccinia viruses, two possibilities need to be considered to explain their origin: (1) that they derive from a natural Orthopoxvirus, or (2) that they are viruses unintentionally created in the uncontrolled manufacturing processes used during the many years of production of smallpox vaccines [99].

Regarding the existence of vaccinia-like viruses circulating in nature, caution is needed in the interpretation of results because the vaccinia-like viruses that have been reported are probably derived from contemporary vaccinia strains. Vaccination against smallpox has been practiced for more than 200 years, significantly increasing during the intensified WHO smallpox campaign (1967–1977). It is known that vaccinated individuals can accidentally transmit vaccinia to contact animals, a situation that is facilitated by the broad host range of vaccinia. Moreover, vaccinia "escapees" have been able to initiate and maintain enzootic cycles in nature, leading to epizootic infections in horses, cattle and other animals caused by vaccinia-like viruses [100–106].

For most of the 19th century smallpox vaccine stocks were maintained by serial arm-to-arm vaccination of children, using different sources of immunizing material, including cases of cowpox, horsepox and even smallpox. This cumbersome procedure began to be replaced as a source of vaccines after 1864 by the use of calves that were vaccinated with matter that had been previously passaged in humans. A further refinement was the adoption of the so-called "animal vaccine" where cowpox matter taken directly from one cow was used to inoculate and serially transfer the vaccine to other cows [107,108]. Those manufacturing developments, and the ever-increasing need to produce large numbers of vaccine doses demanded by the health authorities, led to the establishment of numerous "vaccination farms" around the world, using in many cases pools of different vaccine matter for the inoculation of calves [49,109,110]. Animals were inoculated in multiple sites from which material was collected when the lesions were judged to be "ripe" and the "pulp" ground in a mortar before being suspended in diluent as vaccine "lymph" [1,111–114]. A survey conducted by the World Health Organization (WHO) in 1967 revealed that there were 67 producers of the smallpox vaccine in 45 countries, with most of the producers using calves, a few using sheep or water buffalo, and three of them using tissue culture or embryonated eggs [1]. The WHO then undertook efforts to modernize and standardize the production of smallpox vaccines, resulting in the identification of four strains that were widely used for widespread vaccination: EM-63 (used mostly in the former USSR, said to have derived from a strain from Ecuador), Lister (said to have been originally isolated in Germany but manufactured in England), New York City Board of Health, NYCBH (said to have been brought from England to the US in 1856, and to be the reported parental strain of all US-made smallpox vaccines), and Temple of Heaven or Tiantan (used in China), although many other strains were also used [1,115,116].

Recent genomic analyses have provided additional support for the hypothesis that at least some vaccinia genes derived from horsepox virus. Work published by the group of David Evans, in Canada, suggests that the most probable route by which vaccinia strains may have evolved is from a stock of virus containing an ancestral horsepox-like virus. These findings are based on the identification of three genes (DVX_214 to DVX_216) present in the genome of horsepox virus, but absent in all known vaccinia strains, except for two clones (DPP25 and CL3) of the Dryvax strain [22,116]. Moreover, the phylogenetic analysis of the historical smallpox vaccine used in Brazil until the late 1970s (strain IOC), reportedly derived from the 1866 French Beaugency stock [50], revealed that the strain IOC forms a novel cluster in the phylogeny of the vaccinia lineage, which include vaccinia-IOC, the Brazilian vaccinia field strains Cantagalo and Serro 2 viruses and horsepox virus [20,117].

A more definitive evidence that horsepox virus was used commercially to manufacture smallpox vaccines was the recent finding by our group that an early smallpox vaccine, produced in 1902 by H K Mulford of Philadelphia [118], is phylogenetically closer to horsepox virus rather than to cowpox virus [23]. In addition, the Mulford 1902 vaccine has two deletions in the telomeric regions that are also found in current vaccinia, but not in cowpox or horsepox viruses, suggesting that the horsepox ancestor began to be used as a smallpox vaccine many years before 1902, when the vaccine we characterized was manufactured.

4. Concluding comments

It is rather remarkable that vaccination against smallpox, a preventative intervention that was empirically developed at the end of the 18th century, led to the eradication of one of the most feared diseases at the end of the 20th century. In the absence of laboratory markers of immunogenicity, the smallpox vaccine was developed based on clinical observations, where success was defined by the development in the vaccinated individual of skin lesions considered typical of cowpox, which were carefully described in the contemporary literature. The ultimate efficacy test was the demonstration that vaccination protected the individual from variolation, which basically was a challenge with the variola virus, a procedure that could be practiced in Great Britain until variolation was outlawed by the Vaccination Act of 1840. Since variolation had been the standard of smallpox prevention until vaccination became widely available, it has been argued convincingly that Jenner's smallpox challenge experiments would have passed any hypothetical ethical review conducted at that time [119].

For the first 80–90 years of the vaccination enterprise, the smallpox vaccine was carried and maintained by arm-to-arm inoculation of non-immune children. This cumbersome procedure was not sustainable over time and carried the danger of transmitting diseases such as syphilis. The manufacturing of the vaccine in calves, from the 1860s onwards, was a major advance in the field. However, in the absence of a clear understanding of the principles of virology, which began to be developed only in the 20th century, little attention was paid to careful identification of the source and lineage of the material used to produce the vaccine. In this regard, hearsays and legends replaced well-documented information, with a romantic emphasis in tracing the source of many vaccines to the original cowpox stock of Edward Jenner. In this regard, it is likely that the horsepox origin of many vaccine stocks was hidden to favor the preferred notion that cowpox was the authentic preventative of smallpox. Modern genomic analyses are uncovering little secrets of the past, as is the case of the Chinese smallpox vaccine Tiantan, which has a typical vaccinia genome despite having a history of originating from a case of variola [120–122].

The Tiantan situation may be similar to many other smallpox vaccines used around the world, all having a vague origin and passage history. That same belief was expressed by Edgar Crookshank in 1889: "*While attending at the National Vaccine Establishment of the Local Government Board, I was unable to obtain any exact details, clinical or pathological, of the source of the lymph which was employed there. From my experience of this and other vaccination stations, I found that both official and unofficial vaccinators were completely occupied with the technique of vaccination, to the exclusion of any precise knowledge of the history and pathology of the diseases from which their lymph stocks had been obtained*" [9].

Manufacturing practices in the pre-microbiological era may have led to the intentional or unintentional mixing of vaccine strains, both in human and animal hosts, resulting in horizontal gene transfer and recombination between different Orthopoxviruses [22,123–125]. The current vaccinia strains probably represent the result of multiple evolutionary lineages with sequences derived from different ancestral viruses, including horsepox, which we begin to understand only now. The fact that cowpox viruses have the largest genome among all Orthopoxviruses has led to the suggestion that an ancestral cowpox-like virus is the origin of all Orthopoxviruses that have evolved, in part, by gene loss [126–128]. It is likely that in the relatively long evolutionary history of vaccinia, a few strains were unwittingly selected based on their low reactogenicity, protective efficacy, stability and replication in calves [129].

The phylogenetic analysis of vaccinia and other Orthopoxviruses is complicated by the size and complexity of their genomes [130], with conserved regions and variable telomeres that have resulted from multiple recombinational events. Phylogenetic analysis has been used to classify Orthopoxviruses and define virus species, departing from the traditional approach to name poxviruses based on the first identified animal species in which they produce symptomatic disease [131]. Now we know that cowpox virus is in fact a virus that infects rodents and a provocative suggestion has been made, based on phylogenetic analysis, that the different cowpox virus isolates may actually be reclassified into three to five new species and, for historical reasons, to keep the name of cowpox virus only for the clade that includes the isolates from the United Kingdom [67]. What would be the future phylogenetic understanding of the horsepox viruses remains to be decided if and when new isolates will be obtained from horses, wild rodents or from another hitherto unknown reservoir.

In any case, even if there is increasing evidence that horsepox virus had been used as a vaccine already in Jenner's time, the origin of the different lineages of vaccinia as well as its relationship to cowpox and horsepox viruses is not accurately elucidated yet. The possibility remains that a naturally occurring vaccinia virus, now extinct, was an ancestor to extant vaccinia lineages, perhaps modified by recombinational events with other Orthopoxviruses, especially cowpox and horsepox viruses. Additional clues could be obtained from ongoing work to characterize a number of historical specimens of smallpox vaccines [23].

An exciting development was recently achieved by the group of David Evans, from Canada, with the *in vitro* construction of an infectious horsepox virus from chemically synthesized DNA fragments (David Evans, personal communication) [132]. The resulting virus resembles vaccinia in many respects and its low virulence suggested to the authors that the virus could represent a new generation of safer smallpox vaccines or vaccine vectors.

Authorship

All authors attest they meet the ICMJE criteria for authorship.

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Conflict of interest

None.

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