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The risk of introduction of equine infectious anemia virus into USA via cloned horse embryos imported from Canada

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Abstract

Deriving horse oocytes in the USA is hampered by the lack of abattoirs processing horse carcasses which could provide abundant quantities of ovaries from slaughtered mares. Therefore, several cloning industries in the USA are attempting to import cloned horse embryos from Canada. Like any agricultural commodity, cloned embryos pose a risk of introduction of exotic animal diseases into the importing country. Under such circumstances, risk assessment could provide an objective, transparent, and internationally accepted means for evaluating the risk. This quantitative risk assessment (QRA) was initiated to determine the risk of introduction of Equine infectious anemia virus (EIAV) into USA via cloned horse embryos imported from Canada. In assessing the risk, a structured knowledge base regarding cloning in relation to EIA was first developed. Based on the knowledge base, a scenario tree was developed to determine conditions (with mathematical probabilities) that could lead to the introduction and maintenance of EIAV along the cloning pathway. Parameters for the occurrence of the event at each node were estimated using published literature. Using @RISK software and setting Monte Carlo simulation at 50 000 iterations, the probability of importing an EIAV-infected cloned horse embryo was 1.8×10^{-9} ($R = 1.5 \times 10^{-12}$ to 2.9×10^{-8}). Taking into account the current protocol for equine cloning and assuming the yield of 5 to 30 clones per year, the possible number of EIAV-infected cloned horse embryos ranged from 2.0×10^{-10} to 9.1×10^{-5} (Mean = 1.4×10^{-6}) per year. Consequently, it would take up to 1.5×10^7 ($R = 1.6 \times 10^4$ to 5.1×10^{10}) years for EIAV to be introduced into the USA. Based on the knowledge base and our critical pathway analysis, the biological plausibility of introducing EIAV into USA via cloned horse embryos imported from Canada is extremely low.

Keywords

Cloning; Equine infectious anemia; Embryo; Monte Carlo simulation; Quantitative risk assessment

1. Introduction

Cloning or SCNT (Fig. 1) is a process by which animals are produced asexually. Briefly, in cloning, a differentiated somatic cell is fused (or microinjected) to an oocyte that has had its nucleus removed. Following activation of the oocyte (usually by electrical stimulation), the somatic cell's nucleus is reprogrammed to a pattern of an embryonic genome for directing normal development of the embryo [1,2]. Donor cells can come from a variety of sources,

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but are commonly derived from primary cultures obtained from biopsies of selected adults [2,3]. However, so far, the cytoplasm of mature oocyte is the only one identified with the capacity for reprogramming donor cell nucleus to correctly express genes required for early development [4].

The biggest breakthrough in cloning came in 1996, when ‘Dolly’ the sheep was produced by fusing a cultured adult somatic cell with an enucleated oocyte. Although still quite inefficient [1,5,6], cloning with adult cells offers the advantage of propagating genetically proven animals with known phenotypes [7]. According to Moore & Thatcher [3], this is critical, especially when it may take years to prove the merit of a sire or dam. Additionally, it provides an essentially unlimited supply of donor cells. As a promising tool to help preserve and propagate superior genes, somatic cell cloning has now been successful for producing all livestock species [8].

Owing to its complex nature [9] and variable outcomes [6], many of the peer-reviewed publications on cloning so far report on the ability to generate live animal clones from various donor cell sources and culture conditions. With surviving dam and live offspring as indicators of successful outcomes, these studies (mostly from academic laboratories) usually focus on development of new technologies and observation of developmental abnormalities [1,10,11]. However, there are few studies directly addressing the transfer of adventitious agents [12,13].

In recent years, SCNT technology has assumed greater prominence in the horse industry. Since mares do not respond quite well to superovulation, most studies on in vitro maturation (IVM) use oocytes collected from ovaries of slaughtered mares [11,14]. In the USA, abattoirs processing horse carcasses have been banned; hence, importation of cloned embryos created outside the USA is a viable option to meet the growing demand for equine cloning. Like any other agricultural commodity, cloned embryos pose an uncertain and variable risk for introduction of exotic animal diseases into importing countries [13]. Under such circumstances, risk assessment tools can provide objective, transparent, and internationally accepted means for evaluating the risks involved [15].

The risk assessment process, including the key steps for performing a quantitative risk assessment (QRA), has been outlined. The methodology for quantifying the uncertainty associated with the outcomes has similarly been described [16]. Furthermore, models for the assessment of the animal disease risks associated with the importation of animals [17,18], in vivo derived embryos [19], in vitro produced embryos [20], and other animal products [15], have been presented. In this study, QRA methodology was applied to assess the likelihood of introduction of EIAV into the USA via the importation of cloned horse embryos from Canada. The QRA was based on the prevalence of EIA in Canada. Computer simulations were run to evaluate the probability of EIAV introduction by importing cloned horse embryos contingent to yield 5–30 offspring. This was evaluated in relation to EIA prevalence, sanitary management of somatic cell donor, and cells, oocytes and embryo washing and decontamination procedures, as well as sampling of the media and embryos for their sanitary conditions. The model essentially examined the failure of mitigations either to inactivate, remove, or detect EIAV along the cloning pathway.

2. Materials and methods

2.1. Quantitative risk assessment (QRA) methodology

In the context of animal health, QRA is the process that quantifies the likelihood of the entry, establishment, or spread of an animal pathogen or disease within a territory. Standard epidemiological procedures are utilized to identify hazard scenarios for each step in the

process; mathematical and computing methods are utilized to assess the magnitude of the risk. It is frequently used in assessing the risk associated with importation of agricultural products [21,22], with a prime objective of providing quantifiable scientific information to regulatory authorities.

Our quantitative risk assessment process involved five steps: (a) identifying the hazard (i.e. EIAV); (b) developing a scenario tree which outlines a series of mitigations and all the failures which could occur, culminating in the occurrence of the identified hazard (Fig. 2); (c) gathering and documenting the evidence (see section 2.1.3); (d) developing mathematical equations/functions (Tables 1 and 2); and (e) performing calculations to summarize the likelihood of the hazard occurring (Table 3; Fig. 3).

2.1.1. Assumptions made—Information regarding EIAV transmission through cloned embryo production and transfer is very scarce. Therefore, for this study, relevant information regarding an interaction of EIAV with cloned embryos was extrapolated, mainly from studies conducted on interaction of IVF embryos with lentiviruses, including bovine immunodeficiency virus (BIV), Maedi-Visna virus (MVV), caprine arthritis-encephalitis virus (CAEV), and Human immunodeficiency virus (HIV).

The perceived risk was divided into two sources in the analysis: the health status of donor of the somatic cells; and the health status of mares from which oocytes are collected. As a valuable pedigree animal, the donor of the somatic cells would often be subject to health monitoring and surveillance. Conversely, oocytes, are collected from ovaries of slaughtered randomly selected females at commercial abattoirs with a potential risk of subclinical disease [23]. These potential risks (and respective mitigations) were therefore evaluated separately, until finally combined during fusion of the somatic cell and the cytoplasm. The potential risk after oocyte reconstruction was assessed in reference to internationally accepted standards for processing of embryos [24], and by testing degenerated embryos and embryo wash fluids for EIAV. The risk of introduction of extraneous infectious agents, from co-culture cells and animal products such as serum, for example, was not addressed in this study. It is assumed that the cloning industry has the obligation to conduct procedures according to recommendations and indemnify that media, reagents and equipment, as well as the working environment, are free of pathogens and contaminating microorganisms [25]. Similarly, since cloning is a scrupulous process (and technically demanding), we assumed that human failure (as opposed to process failure) in detecting and removing unclean and infected embryos, was insignificant.

The assessment was conducted up to the entry of at least one EIAV-infected cloned embryo into the USA; spread from this initial point of entry was not considered. As indicated above, a success of cloning is measured by having a cloned offspring. The efficiency of the cloning procedure is therefore critical in determining the number of imported cloned embryos. Based on these facts, and in order to yield a realistic risk estimate, the production of 5–30 cloned offspring was assumed per year. Therefore, the number of imported cloned embryos was modeled based on this contingent (range of clones produced), rather than using a fixed number of imported cloned embryos (as originally suggested).

2.1.2. Development of the scenario tree (critical pathway analysis)—Animal cloning by SCNT is a multi-step process (Fig. 1), which can be grouped into five major steps. These include the selection, isolation and preparation of 1) *donor cells*, and 2) *recipient cells*, and execution of 3) *nuclear transfer*, 4) *embryo culture*, and 5) *embryo transfer* processes. All of these steps have been described in detail elsewhere [9,25–28]. In this study, to model the risk of transmission of EIAV via SCNT, the cloning system was methodically decomposed into its component parts. Then epidemiologic principles

pertaining to EIA were applied and a scenario tree was developed (Fig. 2), represented by a series of risk-associated events (Nodes 1 to 9). From this, conditions (with associated statistical probabilities) that could lead to the introduction and maintenance of EIAV along the cloning pathway, were determined. As such, the model tracks EIAV-infected horse embryos originating from Canada that could introduce EIAV into the USA, provided that a series of mitigations designed to intercept and/or remove the virus all failed.

At each node along the scenario tree, a question was asked (and data collected) with the view of narrowing down the risk through specific mitigation. The accumulation of the answers to these questions (expressed in probability distribution functions) determined the final risk. Quantitative estimates of the risk at each node were obtained from published literature. The ranges of the probabilities were determined by their source documents or their natural domain (all possible values). When only one data source (with a point value) was available, a 95% CI was obtained using the event of interest and respective sample size (EpiInfo 3.5.1). Based on the ranges and estimates, various distribution functions (including Uniform, Pert, Binomial, and Negative binomial) were used to calculate expected values at each node using @Risk version 5.7 (Palisade Corporation, 2010), an add-on to Excel spreadsheet (Microsoft Office Professional Edition, 2010). The sum of the product of the probability distributions, P1 to P3 and P4 to P6, multiplied by P7 to P9, was considered the overall probability of introducing EIAV into USA via cloned horse embryos imported from Canada.

As previously stated [29], data gaps create uncertainty and variability in developing QRA. In order to reduce the uncertainty surrounding the risk estimates, Monte Carlo simulations (a computerized mathematical technique for iteratively evaluating a deterministic model using sets of random values as inputs) were run using @ Risk software. For any variable that has inherent uncertainty, Monte Carlo simulation builds models of possible outcomes by substituting values, randomly from range of possible values (probability distribution). It then calculates results repeatedly (50 000 iterations were used in the present study), each time using a different set of random values from the probability function at each node. The analysis range shows all the possible outcomes, such as going for the most conservative decision, or all possible consequences of middle-of-the-road decisions. This enables the regulatory authorities to compare the acceptable level of risk with its likelihood of occurrence. Furthermore, sensitivity analysis was done to identify the most important inputs in terms of their potential impact on the overall risk estimate. This will open avenues for future surveillance and research endeavors.

2.1.3. Evidence gathering and parameter estimation—Node 1. Is the selected somatic cell donor infected? As an animal with the desirable trait that the cloning procedure is designed to propagate, the source of the somatic cell would often be subject to health monitoring and surveillance [23]. Sticking to the worst case scenario (since the animal health risk is associated with the possibility of bringing intracytoplasmic pathogens within the somatic cell into the recipient oocytes), the probability that the somatic cell donor is affected can be approximated by the disease prevalence in the exporting country. Accordingly, EIA has existed in Canada since the 19th century and is considered endemic in the horse population. Sporadic cases occur throughout Canada, but Northwestern Alberta, Central Saskatchewan, the Eastern Townships in Quebec, the Ottawa Valley, and West-central Manitoba are listed as enzootic areas. The prevalence of infection varies from 0.03 to 0.39% depending on the density of the horse population, the proportion of carrier animals, the insect vector population, and control activities in a particular area [30]. In 2005, the prevalence of EIA in tested horses in Canada was 0.162% [31].

Based on the above evidence, we estimated the probability that donor of the somatic cells is infected equalled the prevalence on the ground, i.e., 0.162% (0.03 to 0.39% -----) (P1).

Node 2. Can diagnostic tests fail to detect an EIAV-infected somatic cell donor? Equine infectious Anemia is a reportable disease in Canada, and the status of horses is determined by their reaction to Coggins test, an official test for EIA [23]. Like any other serological tests, Coggins test can produce false negative results. Nagarajan & Simard [32] previously assessed the sensitivity of this test, using nested PCR as a gold standard. From 88 naturally infected horses with proven proviral DNA in their peripheral blood, 81 (92%; 95% CI = 83.8 to 96.5%) gave a positive reaction to the Coggins test.

Based on the above evidence, we estimated the probability that Coggins test would fail to detect infected donor of somatic cells was most likely 8% (R = 3.5 to 16.2%) ----- (P2).

Node 3. Can tissue culture process remove EIAV from donor cells? Although highly virulent Wyoming and the V26 Japanese strains were studied more extensively, strains of EIAV vary greatly in terms of virulence and ability to grow in cell culture. Most horse-virulent EIAV strains only replicate in primary horse macrophage cultures and cannot replicate in tissue-derived cell lines without extensive adaptation [33]. It has also been ascertained that cellular reservoirs for lentiviruses include cells of the monocyte-macrophage lineage; other cell types do not appear to serve as reservoirs for EIAV [34]. Yet in the horse, adult fibroblasts taken from skin biopsies on the neck or the chest have been used as standard donor cell source for commercial horse cloning [12,26]. However, there still might be a small portion of monocytes lineage cells in the donor cell population. Since the tissue culture condition is tailored to fibroblast cells, it is believed that these monocytes lineage cells in the donor cell population would have been removed after one passage. A 10% probability could be framed as the worst-case scenario for an infected monocyte being used as the donor cell. Additionally, although suppression of retroviral replication is not routinely incorporated in the SCNT procedures, there are several ways of inactivating retroviruses in cell culture.

Harakeh *et al.* [35] reported suppression of virus production and cell fusion in HIV-infected T-lymphocytic cell lines grown in the presence of nontoxic concentrations of ascorbate (Vitamin C). In chronically infected cells expressing HIV at peak levels, ascorbate reduced the levels of extracellular reverse transcriptase (RT) activity (by >99%) and of p24 antigen (by 90%) in the culture supernatant. In freshly infected CD4 cells, ascorbate inhibited the formation of giant-cell syncytia by 93%. Similarly, Sun *et al.* [36] designed several hammerhead ribozymes targeting various sites within retroviral packaging sequences to cleave target RNA in vitro. Based on the p24 antigen and syncytium formation assays, there was an 80–90% reduction in HIV-1 production by the ribozyme starting on Day 6 (of culture) compared to vector-transfected control cells.

Based on the above evidence, we estimated the probability that a somatic cell culture line would be still infected after its treatment during tissue culture with anti-retroviral preparations ranged from 1 to 10% ----- (P3).

Node 4. Is the slaughtered oocyte donor infected? This was discussed under Node 1.

Based on the evidence (see Node 1), we estimated the probability that oocyte donor(s) is/are infected equalled the prevalence on the ground, i.e., 0.162% (0.03 to 0.39% -----) (P4).

Node 5. Could the oocyte be infected? Equine infectious anemia virus (EIAV) has been found in blood, milk, saliva, feces and semen of diseased horses. The virus apparently can cross the placental barrier and cause prenatal fetal infection [13,30,31,37].

In a study to determine whether oocytes taken from ovarian follicles of naturally infected goats carry the proviral CAEV genome, 190 batches of oocytes with intact cumulus cells were compared to 190 batches of oocytes whose cumulus cells had been removed, but taken from follicles of the same ovaries. There were 42/190 batches of oocytes with intact cumulus cells that had the proviral CAEV genome, compared to none of the 190 batches of oocytes without cumulus cells. To confirm that the proviral genome was present in the cumulus cells but not in the oocytes, 586 oocytes from 56 ovaries were separated from their cumulus cells. Molecular analysis demonstrated that none of the 56 batches of the oocytes without cumulus cells (0/56; 95% CI = 0 to 6.4%) was positive, as compared to 22/56 positive batches with cumulus cells. Therefore, despite being surrounded by infected cumulus cells, oocytes were not infected by CAEV, and techniques for removing cells surrounding the oocyte would enable retrieval of CAEV-free oocytes [38].

Based on the above evidence, we estimated the probability that an oocyte could be contaminated in the reproductive tract of an EIAV-infected oocyte donor ranged from 0 to 6.4%

(P5).

Node 6. Can maturation and washing processes remove EIAV from the oocyte?

Oocytes for SCNT are recovered from COCs of abattoir-derived ovaries. After in vitro maturation for 24 h, COCs are exposed to hyaluronidase, and by gentle vortexing, the oocyte is stripped of the surrounding cells. The oocytes are then washed several times in SOF containing fetal calf serum to remove all debris. Furthermore, only ZP-intact oocytes are selected for SCNT [27]. The removal of cumulus cells and multiple washing procedures are believed to remove most of the viral particles [12].

To determine whether successive washing is sufficient to remove virus particle from the surface of the oocyte, Baccetti *et al.* [39] exposed 100 unfertilized human oocytes to infective HIV-1 virus for 24 h. After washing with PBS, all exposed oocytes were negative (0/100; 95% CI = 0 to 3.6%) for HIV-1 by PCR and immunoelectron microscopy. The authors concluded that oocytes derived from HIV-positive women were free of the virus. Terriou *et al.* [40] reported the application of intracytoplasmic sperm injection (ICSI) procedure to help HIV-infected couples have children. According to that report, the largely successful implementation of ICSI procedure for HIV-positive parents demonstrated that the oocyte washing procedure was sufficient to remove HIV from oocytes.

Based on the above evidence, we estimated the probability that in vitro cultured, denuded and washed oocytes would carry EIAV risk ranged from 0 to 3.6% -----
(P6).

Node 7. Can an embryo washing process remove EIAV from the growing embryo?

There are several reports regarding the efficacy of embryo washing to render embryos free of lentiviruses. Ali Al Ahmad *et al.* [41] demonstrated that in vitro CAEV-infected early goat embryos were capable of transmitting CAEV to target cells when embryos were co-cultured with non-infected GSM cells. However, virus particle could be isolated only from up to 50% of embryos and culture media after the fourth washing; the fifth and successive washings were free of infection. Similarly, Vainas *et al.*, [42] demonstrated that embryos produced from maedi visna sero-positive sperm and oocyte donors did not transmit MVV to sero-negative recipients through in vivo-derived micromanipulated (microinjected) embryos.

In the first experiment, 323 zygotes collected from MV seropositive donors were microinjected with MVV before being transferred to 93 seronegative recipients. Fourteen female offspring were studied for viral infection. In another experiment, 89 blastocysts collected from MV seropositive donors were microinjected with gonadal cells and transferred to 43 seronegative ewes. Eleven female lambs were kept for ascertaining viral infection. Overall, 412 embryos collected from seropositive donors were micromanipulated before being transferred into 136 recipients. The recipients were examined (along with 25 female offspring) at 6-mo intervals for the following 36 mo. All were negative for MVV using agar gel immunodiffusion test and indirect ELISA (95.5% sensitivity). In statistical terms, this corresponded to 0% (95% CI = 0 to 2.3%) transmission rate. The authors concluded that sheep zygotes and embryos washed and treated with trypsin seemed to be MVV-free, even if their zona pellucida was subsequently disrupted. Several other reports have similarly indicated that brief exposure to trypsin (during washing) would remove viruses from bovine, ovine, and swine embryos [1,4,43], as well as human embryos and those from other farm animals [44].

Bielanski *et al.* [45] studied the interaction of BIV with in vivo and in vitro produced bovine embryos. In the first experiment, 44 morulae and blastocysts collected from BIV-negative heifers were exposed in vitro to BIV for 24 h, washed and then tested for the presence of the provirus. In the second experiment, 10 to 15 morula and blastocyst-stage embryos (n = 62) fertilized in vitro were transferred to the uterus of each of five BIV infected heifers. Of these, 43 embryos were non-surgically recovered 24 h later and then tested for the presence of the provirus. Although tests were done for the proviral DNA, and not for the viral RNA, all gave a negative result (0/43; 95% CI = 0 to 8.2%); therefore, an infectious form of BIV was not transmitted to the recipients or their offspring.

Based on the above evidence, we estimated the probability that EIAV-infected embryos could still carry infection after washing and trypsin treatment ranged from 0 to 8.2% (most likely = 2.3%) -----
(P7).

Node 8. Can an EIAV-infected embryo develop into an exportable blastocyst? As indicated by studies in other species [46], the proportion of embryos that developed to the blastocyst stage was significantly higher for the control group (29%, 18/62) than for the infected group (13.8%, 17/123). Stringfellow *et al.* [47] also reported reduced development to the blastocyst stage in BVD-exposed COCs (9%) compared to non-exposed COCs (12%). In another study [48], 34.6% of zona pellucida-free embryos exposed to CAEV reached the blastocyst stage, compared to 36.0% in non-inoculated controls. As indicated above, information regarding the interaction of EIAV and cloned (or IVF) embryos was not available. By extrapolating the evidence given above, when compared to control group, the ratio of EIAV-exposed recombined horse oocytes that could reach blastocyst stage ranged from 0.5 to 1. However, under typical culture conditions, a blastocyst development rate of 1.4% (6/423) to 17% (34/200) has been reported for recombined horse oocytes [10].

Based on the above evidence, we estimated the probability that EIAV-infected horse embryos would develop into transferable blastocysts ranged from 0.7 to 17% -----
(P8).

Node 9. Can infection be detected in a developing transferable blastocyst? In the live animal, equine infectious anemia is diagnosed by the Coggins test. The sensitivity of the test, using nested PCR as a reference test, was 92% [32]. Recently, RT-PCR has become a useful tool to detect CAEV RNA in embryo wash fluids [49]. Although standard nucleic acid tests in embryo culture have not been described, a minimum sensitivity of 92% (83.8 to

96.5%) can be achieved by RT-PCR, as is the case with Coggins test in subclinically infected animals.

Based on the above assumption, we estimated the probability that nucleic acid tests would fail to detect EIAV-infected degenerated embryos or EIAV-contaminated wash fluids was 8% (95% CI= 3.5 to 16.2 %.) -----
(P9).

2.1.4. Estimates related to the number of imported blastocysts—As in other agricultural commodities, the magnitude of the perceived risk in SCNT depends on the number of imported cloned embryos. Based on the success of embryo transfer using IVF embryos, Suttmoller & Wrathall [19] previously assumed a yearly import of 200 to 300 IVF embryos in conducting a QRA. In SCNT, the success of cloned blastocysts to yield a live clone is highly variable, due to varying culture conditions [11]. Furthermore, as only a fraction of transferred cloned embryos lead to successful pregnancies and birth of live offspring, the magnitude of imported cloned embryos is contingent upon cloned offspring produced locally. The following section and Table 2 track a cloning pathway so that the number of imported cloned embryos is incorporated into the risk estimate.

Most studies on IVM use oocytes collected from slaughtered mares, either by aspirating follicular contents or by scraping the wall of the follicle. On average, it is possible to recover 3 to 6 immature oocytes per ovary from the ovaries of slaughtered mares. The maturation rate of horse oocytes is also quite variable (averaging, 51.1 to 80.0% [11]). The fusion rate of mature horse oocytes ranged from 66.3% (120/181) [50] to 84% [51]. Rates of cleavage and blastocyst development under a particular laboratory condition were 88.9% (1866/2099) and 4.4% (83/1866), respectively [14,27]. According to Galli *et al.* [14], the transfer of 118 cloned blastocysts resulted in 13 (11%) pregnancies, however, only three (23%) of the pregnant mares produced a foal.

3. Results

Specific inputs for the model and respective calculations are shown (Tables 1 and 2). Results are summarized (Table 3), and some of the selected results are shown (Figs. 3–7). As shown in Table 3, a series of mitigations, including sanitary management of the donor of somatic cells, washing and disinfection procedures of cells, oocytes and embryos according to IETS standards, as well as testing protocols at various levels of the production pathway, were sufficient to reduce the probability of embryo contamination to negligible levels. Accordingly, the probability of introducing EIAV into the USA via cloned horse embryos imported from Canada was 1.8×10^{-9} ($R = 1.5 \times 10^{-12}$ to 2.9×10^{-8}). This is plotted in Fig. 3 for a better graphical presentation. Under this circumstance, approximately 9.7×10^8 cloned horse embryos are required to pose any considerable risk. Interestingly, the mode, which happens to be identical to the maximum number of embryos that would cause a considerable risk, accounted for 25% of the data set (details are provided in Table 3 and Fig. 4).

Conversely, according to the data in Tables 1 and 2, the number of EIAV-infected cloned horse embryos imported into the USA, assuming an importation of 5 to 30 clones' contingent per year was 1.4×10^{-6} (details are provided in Table 3 and Fig. 5). Correspondingly, the number of years it would take before EIAV is introduced into the USA is shown (Fig. 6). On average, an EIAV-contaminated embryo would be imported into the USA once in 10^7 years. Further details are provided in Table 3.

According to our sensitivity analysis (Fig. 7), embryo development to the blastocyst stage, cell culture treatment, and the embryo washing protocol, were among the inputs with high

variability/uncertainty. According to this particular sensitivity analysis, if one of these input variables was changed by one SD, for example by acquiring more data, the corresponding probability of the risk event would change by nearly 40%. Other input variables showing relatively high uncertainty were the efficiency of diagnostic tests to detect sub-clinically infected donor of the somatic cells as well as contaminated embryos/wash fluids, and the status of somatic cell donor. Changing these input variables by one SD would impact the overall probability by 22 to 25%.

4. Discussion

Owing to diversity of systems used for cloned embryo production, standard methods for ensuring that cloned embryos are free of specific pathogens have not been adopted. Also information regarding EIAV transmission through cloned embryo production and transfer is very scarce. In this study, QRA methodology was applied for evaluating the risk of introduction of EIAV into the USA via cloned horse embryos imported from Canada. Clearly, the risk was negligible. In numeric terms, the probability of importing an EIAV-infected cloned horse embryo was 1.8×10^{-9} ; on average, it would take up to 10^7 years (or an average of 9.7×10^8 embryos) for the virus to be introduced. This extremely low risk was mainly due to the low probability of an oocyte being infected, efficiency of oocyte and embryo washing protocols, and low prevalence of EIA in Canada.

Evidence is available regarding the contamination of mouse oocytes by various pathogens [52]. However, despite considerable research in embryo–pathogen interactions in farm animals, there is no firm evidence for transovarial transmission of endogenous retroviruses [19] and, no infectious agent has been retrieved inside the oocyte. In cattle chronically infected with bovine leukosis virus (a retrovirus), for example, there was no evidence for true trans-ovarian infection of oocytes (as distinct from contamination) [53]. Organisms often contaminate follicular fluid, granulosa, and cumulus cells and bind to the ZP (hence are external to the oocyte) before penetrating into the oocyte during fertilization [44]. It seems, therefore, that techniques for removing cells surrounding the oocyte as well as in vitro maturation at 39 °C and washing of the oocyte could account for virus inactivation or removal [46]. Using this principle, Terriou *et al* [40] confirmed that oocyte washing procedure was sufficient to remove HIV from the oocytes, which in turn minimized the risk of embryo infection during in vitro manipulation.

To our knowledge, QRA has not been applied previously to analyze the risk of introducing EIAV via the movement of infected cloned embryos. It is noteworthy that the World Organization for Animal Health (OIE) also concluded that international movement of cloned embryos was unlikely to lead to release of adventitious infectious agents [13]. However, the OIE commented that the extensive manipulation of donor cells, oocytes and embryos involved in cloning by SCNT may exacerbate the risk associated with adventitious agent transfer compared to the risk involved in IVF procedures. The report further specified that no specific health risks have been reported in relation to oocyte enucleation, the fusion of oocyte with somatic cell nucleus, or the injection of somatic cell nucleus directly into the cytoplasm of the enucleated oocyte. In order to minimize any perceived risk, practitioners should espouse the IETS' recommendation that the media in which the embryos are manipulated before compromising the integrity of zona pellucida should be sterile [54]. If this can be ascertained, embryo production by in vitro techniques has low potential for transmitting diseases [25,50], which can be applied in evaluating risk in SCNT [13]. Using this concept, Gregg & Polejaeva [12] recently reviewed the risk of EIAV transmission via SCNT, based on the risk of lentivirus transmission through in vitro embryo production and transfer in various species. They implied that the risk of EIAV transmission via SCNT embryo production in horse was small or absent.

Furthermore, EIA is a reportable disease and subject to control under the Animal Disease and Protection Act and Regulations administered by the Health of Animals Division of Agriculture Canada [31,32]. According to a voluntary control program that was initiated in 1972, accredited veterinarians collect blood samples for serologic (Coggins) test, a highly sensitive test for identifying sub-clinically infected animals. The reactor horses are branded before being slaughtered (with compensation), or quarantined [31]. It can therefore be concluded that the surveillance system in place would allow timely detection of infected somatic cell and/or oocyte donors.

According to our sensitivity analysis (Fig. 7), embryo development, efficiency of cell culture treatment, and embryo washing were among the inputs with high variability/uncertainty, followed by efficiency of diagnostic tests and status of somatic cell donor. Embryo development reflects the efficiency of equine cloning, which is quite variable, depending on the technique used [51]. The overall risk for disease transmission, however, depends on the number of imported blastocysts [25]. The uncertainty surrounding cell culture treatment requires more data and refinement regarding inactivation of EIAV. Embryo washing/with trypsin, which can easily be incorporated into embryo production schemes in SCNT, effectively limits transmission of (viral) pathogens to recipients and offspring when embryos, either collected from infected/exposed donors or exposed to infectious agents in vitro, are transferred to disease-free recipients [25,41,42,48]. Conversely, the efficiency of diagnostic tests and status of somatic cell donor, emphasize our conservative approach in ensuring the health of somatic cell donor. However, as an animal with the desirable trait, the source of the somatic cells would often be subject to sanitary management during its lifetime [23].

In conclusion, this QRA presents the method by which available data on EIA pertaining to the risk of contamination of in-vitro matured, cloned horse embryos were evaluated. According to our QRA, the risk was negligible. However, to substantiate our conclusion it would be necessary to use somatic cells and oocytes from EIAV-infected donors to produce cloned offspring. We emphasize that embryo production by SCNT has some advantages in managing the risk of adventitious agents transfer. In that regard, that each step in the production pathway is technically discrete could provide control points and sufficient time to allow each (intermediate) product to be assessed for its sanitary status. Sanitary management can begin with verification of medical records of the somatic cell donor. Furthermore, samples of the culture media, wash fluids and degenerated embryos would provide insights regarding the infectious milieu to which viable embryos may have been exposed.

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Fig. 1.

Main steps of SCNT: (A) cell donor; (B) oocyte donor; (C) enucleation; (D) cytoplasm; (E) somatic cell culture; (F) injection of a somatic cell between ZP and the membrane of the enucleated oocyte; (G) electrofusion of the oocyte and the cell; (H) embryo clone; (I) embryo transfer into a surrogate dam generating a clone (J). (Source: European Food Safety Authority (EFSA), 2008).

Fig. 2.
A scenario tree for likelihood of introduction of EIAV into USA through cloned horse embryos imported from Canada.

Fig. 3. Probability density function (cumulative distribution) for the risk of introduction of EIAV virus into the USA via cloned horse embryo imported from Canada.

Fig. 4.
Probability distribution for the number of cloned horse embryos that has to be imported into the USA in order to include one EIAV-infected cloned embryo.

Fig. 5. Probability density function for the number of EIAV-infected cloned horse embryos imported into the USA per year.

Fig. 6.
Number of years before an EIAV-infected embryo is introduced into the USA via cloned horse embryos imported from Canada.

Fig. 7.
Sensitivity analysis for the likelihood of introduction of EIAV into the USA via cloned horse embryos imported from Canada.

Table 1

Description of the variables used in the model and the probability distributions used for calculations.

Inputs	Description	Unit	Definition	Calculation *
P1	Is the selected somatic cell donor infected?	$\frac{\text{Infected horses}}{\text{Total horse population}}$	Prevalence	RiskPert(0.03,0.162,0.39)
P2	Would a Coggins test fail to detect infected somatic cell donor?	$\frac{\text{Infected test negative somatic cell donors}}{\text{Infected horses}}$	1-sensitivity	RiskPert(3.5,8.0,16.2)
P3	Can tissue culture process fail to remove the agent?	$\frac{\text{Contaminated cells after processing}}{\text{Infected test negatives}}$	Proportion	RiskUniform(1.0,10.0)
Risk associated with somatic cell donor = P1*P2*P3				
P4	Is slaughtered oocyte donor infected?	$\frac{\text{Infected horses}}{\text{Total horse population}}$	Prevalence	RiskPert(0.03,0.2,0.39)
P5	Could the oocyte be contaminated?	$\frac{\text{Infected oocytes}}{\text{Infected horses}}$	Proportion	RiskUniform(0.0,6.4)
P6	Would oocyte washing fail to remove the agent?	$\frac{\text{Infected oocytes after maturation}}{\text{Infected oocytes}}$	Proportion	RiskUniform(0.0,3.6)
Risk associated with oocyte donors = P4*P5*P6				
P7	Would embryo washing fail to remove the agent?	$\frac{\text{Infected embryos after washing process}}{\text{Total infected embryos}}$	Proportion	RiskPert(0.0,2.3,8.0)
P8	Can an infected embryo develop into an exportable blastocyst?	$\frac{\text{Embryos reaching blastocyst stage}}{\text{Infected embryos after washing process}}$	Proportion	RiskUniform(0.7,17)
P9	Would testing of wash fluids fail to detect infection in a developing blastocyst?	$\frac{\text{Infected test negative embryos}}{\text{Embryos reaching blastocyst stage}}$	Proportion	RiskPert(4.0,8.0,16.0)
Total risk associated with transferable = [(P1*P2*P3) + (P4*P5*P6)]*P7*P8*P9 blastocyst				

* Values are in percentages.

Table 2

Parameters for estimating the number of blastocysts produced.

Probability	Description	Distribution
A	<i>No. oocytes per slaughtered mare</i>	$=RiskUniform(6,12)$
B	<i>Maturation rate of horse oocyte</i>	$=RiskPert(0.51,0.60,0.80)$
C	<i>Fusion rate of mature oocyte</i>	$=RiskUniform(0.66,0.84)$
D	<i>Cleavage rate of fused oocyte</i>	$=RiskBeta(1866+1,(2099-1866)+1)$
E	<i>Blastocyst development rate of cleaved oocyte</i>	$=RiskBeta(83+1,(1866-83)+1)$
F	<i>Pregnancy rate of transferred blastocyst</i>	$RiskBeta(13+1,(118-13)+1)$
G	<i>Pregnancy yielding live (clone) foal</i>	$RiskBeta(3+1,(13-3)+1)$
H	<i>Number[†] of live clones per year</i>	$RiskUniform(5,30)$
Number of blastocysts imported per year = $[1/(F*G)]*H$		

[†] Based on our assumptions

Table 3

The results of 50 000 iterations showing: 1). probabilities at each node; and 2). the probability of introducing ELAV into the USA, number of embryos imported before an ELAV-infected embryo is included, the number of cloned embryos imported per year, the number of infected embryos imported per year, and the number of years it would take for an infected cloned horse embryo to be imported into the USA.

Probabilities	Minimum	5%	Mean	95%	Maximum
P1	3.3×10^{-4}	7.4×10^{-4}	1.8×10^{-3}	2.9×10^{-3}	3.8×10^{-3}
P2	1.9×10^{-3}	3.0×10^{-2}	8.0×10^{-2}	1.3×10^{-1}	1.6×10^{-1}
P3	2.1×10^{-6}	5.0×10^{-3}	5×10^{-2}	9.5×10^{-2}	1.0×10^{-1}
P4	3.3×10^{-4}	7.4×10^{-4}	1.8×10^{-3}	2.9×10^{-3}	3.8×10^{-3}
P5	5.1×10^{-6}	3.2×10^{-3}	3.2×10^{-2}	6.1×10^{-2}	6.4×10^{-2}
P6	3.4×10^{-6}	2.0×10^{-3}	2.0×10^{-2}	3.8×10^{-2}	4.0×10^{-2}
P7	3.7×10^{-4}	7.3×10^{-3}	2.9×10^{-2}	5.5×10^{-2}	7.6×10^{-2}
P8	7.0×10^{-3}	1.5×10^{-2}	8.9×10^{-2}	1.6×10^{-1}	1.7×10^{-1}
P9	3.6×10^{-2}	5.0×10^{-2}	8.6×10^{-2}	1.3×10^{-1}	1.6×10^{-1}
Prob. that imported embryo is contaminated	1.5×10^{-12}	8.0×10^{-11}	1.8×10^{-9}	6.3×10^{-9}	2.9×10^{-8}
Min [§] , no. embryos imported to get one ELAV-infected	1.2×10^4	3.0×10^7	9.7×10^8	2.1×10^9	2.1×10^9
No. cloned embryos imported per year	47	175	749	1861	16253
No. infected embryos per year	2.1×10^{-10}	3.5×10^{-8}	1.4×10^{-6}	5.2×10^{-6}	9.1×10^{-5}
Years until an infected embryo is imported	1.6×10^4	3.1×10^5	1.5×10^7	4.7×10^7	5.1×10^{10}

[§] Minimum no. calculated as a distribution function ($\text{RiskNegbin}(1, 10^{-9}) + 1$, using the calculated probability ($p = 10^{-9}$) and the desired success (s), i.e., the number of infected embryos (s = 1).