

Sensitive methods and improved screening strategies are needed for the detection of pig viruses

To the Editor:

Xenotransplantation using pig cells, tissues or organs may be associated with the transmission of porcine microorganisms, including viruses, to the recipient. At the moment, it is still unclear which porcine microorganisms may infect humans and which are able to induce zoonoses. Hepatitis E virus (HEV), for example, is a porcine virus with known zoonotic transmission. HEV is transmitted by undercooked pig meat or contact to pigs and induces diseases in patients with an underlying liver disease and in immunocompromised patients, but infections of healthy individuals are asymptomatic (for review see¹).

Here, I will concentrate on the porcine cytomegalovirus (PCMV), which is the first porcine virus found transmitted to non-human primates after transplantation of pig organs. In all reported cases, PCMV was detected in the transplant recipients; however, it remains unclear whether the virus infected the non-human primates or was replicating only in the transplants. When transplanting kidneys from PCMV-positive pigs into baboons, a transplant dysfunction was observed and the survival time was reduced to 14.1 days (average of 10 animals) compared with 48.3 days (eight animals) when using kidneys from PCMV-negative pigs.^{2,3} A similar observation was made when kidneys were transplanted into cynomolgus monkeys (28.7, three animals, compared with 9.2 days, five animals).^{3,4} Although in the first experiment the donor pigs were not screened for PCMV before transplantation, the detection of high copy numbers of PCMV in the rejected pig kidneys (up to $5 \times 10^7/300$ ng DNA) clearly demonstrated that PCMV was the reason of kidney dysfunction.² In the other study, the status of positive and negative animals had been demonstrated by PCR testing.⁴

All donor animals should be screened for the presence of various viruses using assays of appropriate sensitivity to reduce the chance of viral transmission. For example, Göttingen Minipigs had been recently used for preclinical islet cell transplantations⁵ and were screened for PCMV. When animals from the production facility were tested using less-sensitive methods in a commercial laboratory and in our laboratory, no PCMV was detected.^{6,7} However, when we used more sensitive PCR-based methods, some Göttingen Minipigs were found positive for PCMV.⁶ No transmission of PCMV was observed when islet cells of these animals were transplanted into cynomolgus monkeys.⁵ Göttingen Minipigs are produced under SPF conditions. The animals are screened twice a year by the company for numerous microorganisms including 27 bacteria, 16 viruses, three fungi and four parasites (<http://www.minipigs.dk/>); however, PCMV is not among these microorganisms.

Although these data show that sensitive assays provide a better chance of virus detection, more sensitive assays are not always enough to detect virus. Recently, in two baboons, who received an

orthotopic pig heart transplant, high titers of PCMV were detected in the blood of the recipients despite the fact that the virus was not found in the donor pigs⁸ [Fiebig et al., in preparation]. In both transplanted animals, PCMV was detected in the pig heart and in several baboon organs (Fiebig et al., in preparation). Whether PCMV infected the baboons or was replicating only in the transplant and disseminated pig cells and whether the short survival times of the baboons was due to PCMV as shown in the above-mentioned trials in baboons² and cynomolgus monkeys⁴ are unclear.

Virus infections are usually characterized by a clear correlation between the virus load and the pathogenic effect of the virus. There are thresholds above which the virus is pathogenic; however, these thresholds are unknown in most cases. They could only be estimated in vivo, for example transmitting serial dilutions of PCMV-infected pig cells into non-human primate recipients. Therefore, it seems logical to reduce the virus load by selection, treatment and/or vaccination best to a minimum (zero) measured by sensitive methods.

Some of the newly developed PCR-based methods are already very sensitive (up to two copies/reaction^{6,7}), and it seems impossible to improve these methods. In this case, the detection strategies should be improved, for example by testing of different source samples. Such samples may be blood, ear biopsies, different organs or non-invasively taken oral and anal swabs.⁹ Whereas for some viruses it is well studied in which organs these viruses replicate best, this is unknown for many other viruses.^{10,11} Therefore, it should be evaluated, in which organs certain viruses can be detected most easily. In our studies, high amounts of PCMV copies were found in the nose, kidney and spleen (J. Denner, E. Plotzki, U & Fiebig, unpublished), and in other studies, PCMV was widely distributed in various organs of the body and mainly affected the immune and respiratory systems¹¹ or the spleen, liver and lung.¹⁰ To screen for vertical viral transmission without sacrificing the donor animal, sibling animals could be used. The presence of horizontally transmitted viruses could employ the use of sentinel animal cohorts. Dependent on the nature of the product, and the manufacture of the product, it is up for discussion whether negative testing of the xenotransplantation product, for example the islet cell preparation, is sufficient even in the case of positive testing of the animals.

Another way to improve the screening strategies is to test at different time points. The optimal time point depends on the virus. When the virus replication increases steadily, it seems best to screen immediately before transplantation. If the virus goes into latency, it is effective to screen as soon as possible after infection. As we do not know when a viral infection occurs, the timing post-infection cannot be calculated. For congenitally transmitted viruses, testing shortly after birth

or after maternal antibodies waned can be suggested. However, the best way would be to test repeatedly at different time points.

As latent PCMV infections as well as other virus infections are difficult to detect, in addition to PCR-based methods, immunological methods indicating infection by detection of antibodies against the viruses should be used more often. To improve immunological testing, we developed recently Western blot assays for PERV,^{12,13} HEV,^{8,14,15} porcine lymphotropic herpesvirus,¹⁶ as well as PCMV.¹⁷

Most of the porcine microorganisms are not adapted to humans, and infection may be prevented by the innate and adaptive immune system, by intracellular restriction factors as well as by the absence of specific receptors on human cells. In the first clinical xenotransplantations (more than 200 cases, for review see^{18,19}), and in two recent pig islet cell transplantations to treat diabetes in humans in New Zealand and Argentina,^{12,20} no porcine microorganisms including porcine endogenous retroviruses (PERVs) were found transmitted. Auckland Island pigs were used in these two studies, and the herd, each donor pig and the transplants were tested negative for 24 bacteria, viruses and toxoplasma.^{12,20} A prospective preclinical islet cells transplantation was performed beforehand in cynomolgus monkeys, none of the animals was infected with PERV or other viruses.²¹

At present, only a small number of preclinical^{5,8,15,21,22} and clinical^{12,20} xenotransplantations using well-characterized donor pigs have been performed, and only in two of the preclinical cases, transmission of PCMV was observed⁸ [Fiebig et al., in preparation]. These findings will facilitate the improvement of testing strategies in future. Our attempts to develop new sensitive methods and our screening of donor pigs and non-human primates in preclinical xenotransplantation trials suggest that highly sensitive methods, selection of the right source material and selection of the right time point of testing are equally important for an effective detection of porcine viruses.

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