PART 1 (COUNCIL DECISION 2002/813/EC)

SUMMARY NOTIFICATION INFORMATION FORMAT FOR THE RELEASE OF GENETICALLY MODIFIED ORGANISMS OTHER THAN HIGHER PLANTS IN ACCORDANCE WITH ARTICLE 11 OF DIRECTIVE 2001/18/EC

In order to tick one or several possibilities, please use crosses (meaning x or X) into the space provided as (.)

A. General information

1. Details of notification

(a)	Member State of notification	**Slovenia**
(b)	Notification number	**B/SI/14/01**
(c)	Date of acknowledgement of notification	**24. 10. 2014**
(d)	Title of the project	**The combination of surgery or electrochemotherapy and gene electrotransfer of naked plasmid DNA encoding canine interleukin 12 for the treatment of oral and skin tumors in dogs**
(e)	Proposed period of release	**From 1/1/2015 until 31/12/2020**

2. Notifier

Name of institution or company:

University of Ljubljana, Veterinary faculty, Gerbičeva 60, Ljubljana, Slovenia

3. GMO characterisation

(a) Indicate whether the GMO is a:

viroid	(.)
RNA virus	(.)
DNA virus	(.)
bacterium	(.)
fungus	(.)
animal:	
- mammals	(.)
- insect	(.)
- fish	(.)
- other animal	(.)

specify phylum, class

Plasmid pCMVcaIL12 is an artificially created plasmid and it doesn't exist in nature. Plasmid is intended for research use only. Plasmid does not have any characteristics of a living organism, therefore it cannot be called "genetically modified organism" per se.

(b) Identity of the GMO (genus and species)

Not relevant. The plasmid DNA does not have a taxonomic classification.

(c) Genetic stability – according to Annex IIIa, II, A(10)
Annex IIIa, II, A(10): Verification of the genetic stability of the organisms and factors affecting it.

Beside the gene for IL12, pCMVcaIL12 plasmid also contains origin of replication (Ori) and antibiotic resistance gene. The amplification of plasmid is because of Ori possible only in *E.coli*. Antibiotic resistance gene can also amplify only in *E.coli* JM107, because the resistance gen has its own promoter specific only for *E.coli*. That is why the expression of resistance gene is not possible in mammalian cells. The bacteria are not present in the tissue we are going to transfect with the plasmid. It has been proved that plasmid is not transferred into the bacteria that are normally present on the skin of dogs (Krhač Levačić 2013, Release into the environment no. 35419-1/2009).

The plasmid will be administered peritumorally into intact skin or mucous membrane. This will be directly followed by electroporation using sterile applicator electrodes. Injection site, where plasmid DNA and electric pulses will be administered, will be disinfected according to the standards for surgical disinfection before the application. After the administration we will bandage the wound if on skin and the dogs will receive the Elizabethan collar. The wound will remain bandaged for 7-10 days.
Before taking the dog home, the owners will get precise information about the wound care and the amount of time the dogs will have to wear an Elizabethan collar. The dogs will have to wear the collar for 3 weeks because after that time we did not detect plasmid DNA on the site of application anymore (Release into the environment no. 35419-1/2009).

The entry of plasmid DNA into skin cells and consequently the entry in blood circulation and other organs from where it could spread into the environment is practically impossible. If this would happen the possibility of plasmid transfection into other organisms and successful expression of the transgene especially in toxic amounts is negligible. That is because the efficiency of naked plasmid DNA transfection without any additional physical or chemical methods is low (Čemažar M et al., 2002).

4. Is the same GMO release planned elsewhere in the Community (in conformity with Article 6(1)), by the same notifier?
Yes (.) No **(x)**
If yes, insert the country code(s) …

5. Has the same GMO been notified for release elsewhere in the Community by the same notifier?
Yes (.) No **(x)**

If yes:

- Member State of notification …
- Notification number B/../../…

Please use the following country codes:
Austria AT; Belgium BE; Germany DE; Denmark DK; Spain ES; Finland FI; France FR; United Kingdom GB; Greece GR; Ireland IE; Iceland IS; Italy IT; Luxembourg LU; Netherlands NL; Norway NO; Portugal PT; Sweden SE

6. Has the same GMO been notified for release or placing on the market outside the Community by the same or other notifier?

Yes (.) No (x)

If yes:

- Member State of notification …
- Notification number B/../../…

7. Summary of the potential environmental impact of the release of the GMOs.

Plasmid pCMVcaIL12 is a DNA molecule which has no characteristics of a living organism or virus and cannot be considered as a GMO. All genes, which allow mobilization of the plasmid, are deleted, and therefore it cannot be transferred between transfected and not-transfected cells. It cannot replicate in mammalian organism and it doesn't insert into host's genome.

In instance, that plasmid is released into the environment; possibility of transfer into human or animal organism is negligible small, especially in quantities which would allow expression of gene product in any significant concentrations. Vertical transmission of plasmid is not possible. The probability of bacterial uptake of the plasmid, facilitating replication, is very small. Bacteria cannot express gene product. Transfer of plasmid between bacteria is not possible. Replication of plasmid is possible only in *E. coli* due to specific origin of replication.

Plasmid pCMVcaIL12 carries genes, encoding resistance to antibiotic. Transcription of these genes is possible only in *E. coli*, due to specific promoter. Therefore, expression of resistance is not possible in mammalian cells.

The potential environmental risk of pCMVcaIL12 is therefore considered to be minimal or negligible and acceptable in view of the potential clinical benefit to dogs with cutaneous and oral tumors.

B. Information relating to the recipient or parental organism from which the GMO is derived

1. Recipient or parental organism characterisation:

(a) Indicate whether the recipient or parental organism is a:

(select one only)

viroid (.)

RNA virus (.)
DNA virus (.)
bacterium (.)
fungus (.)
animal
- mammals (.)
- insect (.)
- fish (.)
- other animal (.)
(specify phylum, class) …

other, specify

pCMVcaIL12 is an artificially created bacterial plasmid which does not exist in the natural environment and does not have a taxonomic classification.

2. Name

(i)	order and/or higher taxon (for animals)	…
(ii)	genus	…
(iii)	species	…
(iv)	subspecies	…
(v)	strain	…
(vi)	pathovar (biotype, ecotype, race, etc.)	…
(vii)	common name	**pCMVcaIL12**

3. Geographical distribution of the organism

(a) Indigenous to, or otherwise established in, the country where the notification is made:
Yes (.) No (.) Not known (.)

It does not have natural environment and is used in research laboratories worldwide.

(b) Indigenous to, or otherwise established in, other EC countries:
(i) Yes (.)

If yes, indicate the type of ecosystem in which it is found:

Atlantic ..
Mediteranean ..
Boreal ..
Alpine ..
Continental ..
Macaronesian ..

(ii) No (.)
(iii) Not known (.)

It does not have natural environment and is used in research laboratories worldwide.

(c) Is it frequently used in the country where the notification is made?
Yes (.) No **(x)**

(d) Is it frequently kept in the country where the notification is made?
Yes (.) No **(x)**

4. Natural habitat of the organism

It is artificially developed and does not have natural habitat.

(a) If the organism is a microorganism

water	(.)
soil, free-living	(.)
soil in association with plant-root systems	(.)
in association with plant leaf/stem systems	(.)
other, specify	…

(b) If the organism is an animal: natural habitat or usual agroecosystem:
…

5. (a) Detection techniques

Polymerase Chain Reaction (PCR) assays and qRT-PCR

(c) Identification techniques

Polymerase Chain Reaction (PCR) assays and qRT-PCR

6. Is the recipient organism classified under existing Community rules relating to the protection of human health and/or the environment?
Yes (.) No **(x)**
If yes, specify
…

7. Is the recipient organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?
Yes (.) No **(x)** Not known (.)

If yes:

(a) to which of the following organisms:

humans	(.)
animals	(.)

plants (.)
other (.)

(b) give the relevant information specified under Annex III A, point II. (A)(11)(d) of Directive 2001/18/EC

…

8. Information concerning reproduction

(a) Generation time in natural ecosystems:

The pCMVcaIL12 is capable of replication in permissive bacteria *E.coli*. In laboratory cultures, doubling times of 24-71 minutes have been reported (Bremer H et al, 1986).

(b) Generation time in the ecosystem where the release will take place:

Plasmid pCMVcaIL12 can only amplify in *E.coli* due to the Ori site. These bacteria are not normally present in the tissue we are going to transfect.

(c) Way of reproduction: Sexual .. Asexual **(x)**

Replication in bacteria (possible only in *E. coli*)

(d) Factors affecting reproduction:

Reproduction (i.e. replication) is possible only in *E. coli*, due to specific origin of replication (Ori). pCMVcaIL12 plasmid also contains an antibiotic resistance gene. Resistance gene can amplify only in *E.coli* JM107, because the resistance gen has its own promoter specific only for *E.coli*. That is why the expression of resistance gene is not possible in mammalian cells.
In addition, the injected plasmid DNA would be rapidly degraded in blood and other tissues, if it would disseminated to these tissues. Data demonstrated that in muscles most of the DNA is degraded in first 30 min after electroporation (Bureau et al, 2004). In blood, the half-life of plasmid DNA is less than 5 min (Lew et al, 1995, Parker et al, 1995). Study quantifying the amount of plasmid DNA incorporated in cationic-lipid in the gonads demonstrated that no plasmid DNA could be detected in the gonads using available methods (PCR) (Vilalta et al, 2005).

These bacteria are not normally present in the tissue we are going to transfect. It has also been proved that the plasmid is not transferred into the bacteria that are normally present on the skin of dogs (Krhač Levačić 2013, Release into the environment no. 35419-1/2009). In the unlikely case, that the plasmid comes into contact with intestinal microflora, uptake of these bacteria is very low (uptake is possible only into competent strains of bacteria, which do not appear in natural environment). The amount of plasmid DNA will depend on tumor size and will not exceed 2mg for one application.

9. Survivability

(a) ability to form structures enhancing survival or dormancy:

(i)	endospores	(.)
(ii)	cysts	(.)
(iii)	sclerotia	(.)
(iv)	asexual spores (fungi)	(.)
(v)	sexual spores (funghi)	(.)
(vi)	eggs	(.)
(vii)	pupae	(.)
(viii)	larvae	(.)
(ix)	other, specify	

It does not form any structures, listed above.
The pCMVcaIL12 is not known to form any of the structures listed above. Instances of inadvertent environmental release would generally be expected to lead to rapid degradation of the pDNA due to the abundance of deoxyribonucleases in the natural environment. Because the dog presents closed system the risk for release of the plasmid in the enviroment is minimal.

(b) relevant factors affecting survivability:

Instances of inadvertent environmental release would generally be expected to lead to rapid degradation of the pDNA due to the abundance of endonucleases in the natural environment.

10. (a) Ways of dissemination

Possibility that the plasmid will be taken up by competent *E. coli*, which could replicate it, is negligibly small. Vertical transmission is not possible.

(b) Factors affecting dissemination

Plasmids pCMVcaIL12 have Ori site which is specific for *E. coli*. These bacteria are not common on canine skin and oral tumors in dogs, where the plasmid will be administered. Plasmid DNA cannot replicate in mammalian organism and it doesn't insert into host's genome.

Introduction of pCMVcaIL12 into close proximity of tumor will be facilitated using electroporation of host tissue (skin and oral mucosae), which allows local transfection of only electroporated tissue. Application of naked DNA, without use of electric pulses, is a very inefficient method of transfection, enabling poor transgene expression (transfection efficiency of less than 1%). Use of electric pulses (i.e. gene electrotransfer, electroporation) significantly increases transfection efficiency (Čemažar, 2002). Naked DNA, which doesn't cross cell membrane and stays in extracellular matrix, is rapidly degraded with endonucleases. Therefore, possibility of plasmid entry into circulation and transfer into other organs, which would allow dissemination into environment (e.g. respiratory, digestive, urinary, reproductive tract), is negligible small.

11. Previous genetic modifications of the recipient or parental organism already notified for release in the country where the notification is made (give notification numbers)

None.

C. **Information relating to the genetic modification**

1. Type of the genetic modification

(i)	insertion of genetic material	(x)
(ii)	deletion of genetic material	()
(iii)	base substitution	(.)
(iv)	cell fusion	(.)
(v)	others, specify …	

2. Intended outcome of the genetic modification

Plasmid pCMVcaIL12, which is not a GMO per se, is an artificially created plasmid.

3. (a) Has a vector been used in the process of modification?

Yes (.) No **(x)**

If no, go straight to question 5.

(b) If yes, is the vector wholly or partially present in the modified organism?

Yes (.) No (.)

If no, go straight to question 5.

4. If the answer to 3(b) is yes, supply the following information

(a) Type of vector

plasmid	(.)
bacteriophage	(.)
virus	(.)
cosmid	(.)
transposable element	(.)
other, specify …	

(b) Identity of the vector

…

(c) Host range of the vector

…

(d) Presence in the vector of sequences giving a selectable or identifiable phenotype

Yes (.) No (.)

antibiotic resistance (.)
other, specify …

Indication of which antibiotic resistance gene is inserted
…

(e) Constituent fragments of the vector
…

(f) Method for introducing the vector into the recipient organism

(i) transformation (.)
(ii) electroporation (.)
(iii) macroinjection (.)
(iv) microinjection (.)
(v) infection (.)
(vi) other, specify …

5. If the answer to question B.3(a) and (b) is no, what was the method used in the process of modification?

(i) transformation (.)
(ii) microinjection (.)
(iii) microencapsulation (.)
(iv) macroinjection (.)
(v) other, specify

pCMVcaIL12 was constructed using standard cloning methods.

6. Composition of the insert

(a) Composition of the insert

The coding sequence for canine IL-12 was cut out of the pCDNA 3.1.ZeosccaIL12 plasmid with restriction enzymes ApaI and NheI and directionally cloned in a vector pVax (Invitrogene). Plasmid pCMVcaIL12 is described in patent application, number UK IPO 1417148.2.

(b) Source of each constituent part of the insert

Data is not available, because it is commercially available product.

(c) Intended function of each constituent part of the insert in the GMO

- **CMV promotor: Expression of the canine IL12 is under control of CMV (cytomegalovirus) promotor and enhancer**

- **caIL12 fusion gene: canine IL12 gene from start to stop codon. Size: 1594 bp**

- **BGH polyA: Bovine growth hormone polyadenylation signal**
- **pUC Ori: is an *E.coli* origin of replication**
- **KanR (kanamycin resistance gene): The kanamycin resistance gene allows the selection of bacteria carrying the pCMVcaIL12 plasmid**

(e) Location of the insert in the host organism

-	on a free plasmid	**(x)**
-	integrated in the chromosome	(.)
-	other, specify …	

(f) Does the insert contain parts whose product or function are not known?

Yes (.) No (x)

If yes, specify …

D. Information on the organism(s) from which the insert is derived

1. Indicate whether it is a:

viroid		(.)
RNA virus		(.)
DNA virus		(.)
bacterium		(.)
fungus		(.)
animal		
-	mammals	**(X)**
-	insect	(.)
-	fish	(.)
-	other animal	(.)
	(specify phylum, class)	…
other, specify		

2. Complete name

(i)	order and/or higher taxon (for animals)	Mammalia, Carnivora, Canidae
(ii)	family name for plants	…
(iii)	genus	Canis
(iv)	species	C. lupus
(v)	subspecies	C. lupus familiaris
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	Dog

3. Is the organism significantly pathogenic or harmful in any other way (including its extracellular products), either living or dead?
Yes (.) No **(x)** Not known (.)
If yes, specify the following:

(b) to which of the following organisms:

humans (.)
animals (.)
plants (.)
other ..

(b) are the donated sequences involved in any way to the pathogenic or harmful properties of the organism
Yes (.) No **(x)** Not known (.)

If yes, give the relevant information under Annex III A, point II(A)(11)(d):
…

4. Is the donor organism classified under existing Community rules relating to the protection of human health and the environment, such as Directive 90/679/EEC on the protection of workers from risks to exposure to biological agents at work?
Yes (.) No **(x)**
If yes, specify …

5. Do the donor and recipient organism exchange genetic material naturally?
Yes (.) No **(x)** Not known (.)

E. Information relating to the genetically modified organism

1. Genetic traits and phenotypic characteristics of the recipient or parental organism which have been changed as a result of the genetic modification

(a) is the GMO different from the recipient as far as survivability is concerned?
Yes (.) No () Not known (X)
Specify …

pCMVcaIL12 doesn't exist in natural environment. The potential for survival of the pCMVcaIL12 in the natural environment as a consequence of the intended use is considered remote.

(b) is the GMO in any way different from the recipient as far as mode and/or rate of reproduction is concerned?
Yes (.) No **(x)** Unknown (.)
Specify …

(c) is the GMO in any way different from the recipient as far as dissemination is concerned?
Yes (.) No **(x)** Not known (.)

Specify …

(d) is the GMO in any way different from the recipient as far as pathogenicity is concerned?
Yes (.) No **(x)** Not known (.)
Specify …

The pathogenicity of pCMVcaIL12 in humans and animals is not known. The use of IL12 gene therapy in human and animal clinical studies has not shown any systemic toxicity (Daud A et. al., 2008, Pavlin D et al., 2011; Čemažar et al., 2011; Release into the environment no. 35419-1/2009).

2. Genetic stability of the genetically modified organism
See A (3) (c) above

3. Is the GMO significantly pathogenic or harmful in any way (including its extracellular products), either living or dead?
Yes (.) No **(x)** Unknown (.)

(a) to which of the following organisms?

humans (.)
animals (.)
plants (.)
other …

(b) give the relevant information specified under Annex III A, point II(A)(11)(d) and II(C)(2)(i)
Annex III A, point II(A)(11)(d): Pathogenicity: infectivity, toxigenicity, virulence, allergenicity, carrier (vector) of pathogen, possible vectors, host range including non-target organism. Possible activation of latent viruses (proviruses). Ability to colonise other organisms.…
Annex III A, point II(C)(II)(i):

Gene therapy of skin and oral tumors in dogs will lead to expression of encoded product (caIL12), which will have therapeutic antitumor effect. IL12 stimulates proliferation of peripheral mononuclear blood cells, activation of natural killer cells and stimulation of interferon-gamma production. High amount of IL-12 can cause side effect, such as fever, nausea (Leonard et al, 1997). However, with doses of plasmid, which will be used, it is not possible to reach toxic concentrations of IL12 in any human or animal organism (Pavlin et al, 2008, Daud et al 2008; Pavlin et al 2011; Čemažar et al 2011; Release into the environment no. 35419-1/2009).

4. Description of identification and detection methods

(a) Techniques used to detect the GMO in the environment

Polymerase Chain Reaction (PCR) assays and qRT-PCR

(b) Techniques used to identify the GMO

Polymerase Chain Reaction (PCR) assays and qRT-PCR

F. Information relating to the release

1. Purpose of the release (including any significant potential environmental benefits that may be expected)

The purpose of release is clinical trial, studying antitumor effects of electrogene therapy using plasmid pCMVcaIL12 in dogs with skin and oral tumors.

Patients, which will be included into study, will be released to home environment after performed gene therapy. The purpose of our study is to achieve antitumor effect of gene therapy enabling improvement of quality of life and prolonging survival in treated subjects and to integrate gene therapy into veterinary medicine.

2. Is the site of the release different from the natural habitat or from the ecosystem in which the recipient or parental organism is regularly used, kept or found?

Yes (.) No (.)

If yes, specify

Not relevant. There is no natural habitat or ecosystem for the plasmid pCMVcaIL12.

3. Information concerning the release and the surrounding area

(a) Geographical location (administrative region and where appropriate grid reference):

Not relevant - the extent of planned clinical trial is very limited to Slovenia. We except animals form different locations in Slovenia. Therefore geographical locations of release cannot be determined in advance.

(b) Size of the site (m^2): … m^2
(i) actual release site (m^2): … m^2
(ii) wider release site (m^2): … m^2

Not relevant, cannot be defined.

(c) Proximity to internationally recognised biotopes or protected areas (including drinking water reservoirs), which could be affected:

Not relevant (see below).

(d) Flora and fauna including crops, livestock and migratory species which may potentially interact with the GMO

Not relevant (see below).

Sections (b) - (d) are not considered relevant due to the fact, that the release is not intended beyond very limited extent (treatment of individual trial subjects). Gene therapy will be carried out at Veterinary faculty, Clinic for companion animals, Cesta v Mestni log 47, Ljubljana. Dogs, which will be included into clinical trial, will receive application of plasmid peritumorally in unharmed skin or oral mucosae. The application of electric pulses will follow. Dose of used plasmid will vary according to size of treated tumor, and will not exceed 2 mg of plasmid per one treatment.

Our patients will be mostly older dogs, which are physically impaired due to their age and disease (advanced stages of different types of cancer), and therefore cannot tolerate normal physical exercise. Daily walks are usually carried out only on short distances with animals on leash.

4. Method and amount of release

   (a) Quantities of GMOs to be released:
   Up to maximum amount of 200 µg of plasmid per patient.

   (b) Duration of the operation:
   1.1.2015 – 31.12.2020

   (c) Methods and procedures to avoid and/or minimize the spread of the GMOs beyond the site of the release

The plasmid will be administered peritumorally in intact skin or mucous membrane, this will be directly followed by electroporation using a sterile applicator electrodes. Injection site, where plasmid DNA and electric pulses will be administered, will be disinfected according to the standards for surgical disinfection before the application. After the administration we will bandage the wound if on skin and the dogs will receive the Elizabethan collar. The wound will remain bandaged for 7-10 days.

Before taking the dog home, the owners will get precise information about the wound care and the amount of time the dogs will have to wear an Elizabethan collar. The dogs will have to wear the collar for 3 weeks because after that time we did not detect plasmid DNA on the site of application anymore (Release into the environment no. 35419-1/2009).

5. Short description of average environmental conditions (weather, temperature, etc.)
   …
   The pCMVcaIL12 will be administered to trial subjects at clinical sites in an enclosed facility at ambient temperature.

6. Relevant data regarding previous releases carried out with the same GMO, if any, specially related to the potential environmental and human health impacts from the release.

No data available because no previous releases have been carried out with the same plasmid.

G. Interactions of the GMO with the environment and potential impact on the environment, if significantly different from the recipient or parent organism

1. Name of target organism (if applicable)

(i)	order and/or higher taxon (for animals)	**Mammalia, Carnivora, Canidae**
(ii)	family name for plants	…
(iii)	genus	**Canis**
(iv)	species	**C. lupus**
(v)	subspecies	**C. lupus familiaris**
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	**Dog**

2. Anticipated mechanism and result of interaction between the released GMOs and the target organism (if applicable)

Gene therapy of peritumoral tissue in dogs will lead into expression of encoded product (caIL12), which will have therapeutic antitumor effect. IL12 stimulates proliferation of peripheral mononuclear blood cells, activation of natural killer cells and stimulation of interferon-gamma production.

3. Any other potentially significant interactions with other organisms in the environment

Other potentially significant interactions with other organisms in the environment are not expected for the reasons, given under A7 in B 8-10.

4. Is post-release selection such as increased competitiveness, increased invasiveness for the GMO likely to occur?

Yes (.) No (x) Not known (.)

Give details

…

5. Types of ecosystems to which the GMO could be disseminated from the site of release and in which it could become established

Plasmid pCMVcaIL12 (not a GMO) could potentially disseminate into soils due to accidents in transport or improper handling. The possibility of dissemination and establishment in soil or other ecosystems in these cases is considered remote, due to plasmid properties, described above, and presence of naturally occurring endonucleases in the environment.

6. Complete name of non-target organisms which (taking into account the nature of the receiving environment) may be unintentionally significantly harmed by the release of the GMO

(i)	order and/or higher taxon (for animals)	**Primates, Hominidae**
(ii)	family name for plants	…

(iii)	genus	**Homo**
(iv)	species	**H. sapiens**
(v)	subspecies	…
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	**Human**

(j)	order and/or higher taxon (for animals)	
(ii)	family name for plants	**Enterobacteriaceae**
(iii)	genus	**Escherichia**
(iv)	species	***E. coli***
(v)	subspecies	…
(vi)	strain	…
(vii)	cultivar/breeding line	…
(viii)	pathovar	…
(ix)	common name	***E. coli***

7. Likelihood of genetic exchange in vivo

(a) from the GMO to other organisms in the release ecosystem:

The likelihood of genetic exchange between pCMVcaIL12 and any non-target organism is considered remote for the reasons stated under A7 and B 8-10.

(b) from other organisms to the GMO:

Not relevant, because the plasmid is not a GMO.

(d) likely consequences of gene transfer:

Human - in case of transfection of any human tissue and consequently expression of transgene, the level of expression would be very low, due to low doses of used plasmid and low efficiency of transfection. With doses of plasmid, which will be used, it is not possible to reach toxic concentrations of IL12 in any human or animal organism.

***E. coli* - in the unlikely case the plasmid will come into contact with competent bacteria in urinary or digestive tract and dissemination into environment. But plasmid cannot be transferred between transfected and not-transfected bacterial cells. Replication of plasmid is possible only in *E. coli* due to specific replication origin. Expression of transgene product is not possible in bacterial cells. The uptake of plasmid in the absence of kanamycin will not have any advantage for growth and will lead to loss of plasmid.**

8. Give references to relevant results (if available) from studies of the behaviour and characteristics of the GMO and its ecological impact carried out in stimulated natural environments (e.g. microcosms, etc.):

No studies of pCMVcaIL12 have been performed in simulated natural environments.

9. Possible environmentally significant interactions with biogeochemical processes (if different from the recipient or parental organism)

No significant interactions with biogeochemical processes are anticipated.

H. Information relating to monitoring

1. Methods for monitoring the GMOs

2. Methods for monitoring ecosystem effects
…

3. Methods for detecting transfer of the donated genetic material from the GMO to other organisms
…

4. Size of the monitoring area (m^2)
… m^2

5. Duration of the monitoring
…

6. Frequency of the monitoring
…

Dogs, included into clinical trial, will be examined once a week at the Veterinary faculty, Clinic for companion animals. Examination will include taking blood samples and skin smears for the presence of plasmid DNA.

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I. Information on post-release and waste treatment

1. Post-release treatment of the site

No post-release treatment of the site is necessary.

2. Post-release treatment of the GMOs

The site of application of plasmid and electric pulses will be protected from any possible contact with humans or animals with adhesive tapes, bandages or similar mechanical protection for 7-10 days. The dogs will have to wear the Elizabethan collar for 3 weeks because after that time we did not detect plasmid DNA on the site of application

anymore (Release into the environment no. 35419-1/2009). The owners will be instructed to make daily walks with dogs on leashes and on short distances in close proximity of homes and to avoid any contact with other animals.

Disposal of used electrodes, scalpels syringes, gloves or other items that may potentially contain residual amounts of plasmid will be performed in accordance with institutional procedures of the clinical site.

3. (a) Type and amount of waste generated

The amount of plasmid DNA in disposable medical equipment (electrodes, scalpels, syringes, suture material, needles, surgical gloves, etc) that may contain plasmid DNA will not exceed 200 μg.

3. (b) Treatment of waste

All disposable material, which will come into contact with patients, will be placed into plastic containers (sharps container). All waste will be incinerated in accordance with guidelines for good laboratory practice and according to European standard 17025. Disposal of waste will be performed by accredited contractor Saubermacher Slovenija d.o.o. Surgical instruments, which will come into contact with patients, will be disposed into plastic containers with disinfection fluid (Sekusept plus, 1% solution) and promptly autoclaved. Urine and excrement of treated dogs won't undergo any special treatment, since treated animals do not excrete any kinds of toxins, allergens or other potentially harmful substances.

J. Information on emergency response plans

1. Methods and procedures for controlling the dissemination of the GMO(s) in case of unexpected spread

Personnel, which will cooperate in the study, will be notified about procedures concerning cleaning and disposal of plasmid solution in case of unintentional spilling of the liquid (mechanical removal of DNA solution, disposal in waste container for hazardous material, use of personal protective equipment).

In case that one of dogs, which will be included in the study, runs away and gets lost either from Veterinary faculty, or from its owner, leader of the project should be notified. He/she will activate personnel of Clinic for companion animals to start searching in the neighborhood of Clinic. If the patient isn't found, animal shelters, Veterinary administration of Republic of Slovenia and Ministry of the environment and spatial planning will be informed. Due to the limited extent of our clinical study, protective measures and specific properties of our patients (pets with at least some obedience training), we think that possibility of any harmful effects on people or environment is negligible. Therefore a notification of above mentioned institutions is only informative.

2. Methods for removal of the GMO(s) of the areas potentially affected

Material that came into contact with plasmid solution will be mechanical removed. In addition, spilled liquids will be absorbed with common absorbent materials and placed in appropriate containers for disposal.

3. Methods for disposal or sanitation of plants, animals, soils, etc. that could be exposed during or after the spread

Disposal or sanitation of plants, animals, soils, etc. exposed to the product is not necessary due to the low risk of resultant harm.

4. Plans for protecting human health and the environment in the event of an undesirable effect

Because the used plasmid does not appear to pose a significant risk to human health or the environment, specific plans for protection have not been prepared.

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