



9 December, 2016

Regulations Review

Office of the Gene Technology Regulator (MDP 54)  
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**Submission to the 2016 Technical Review of the Gene Technology Regulations 2001**

Responding to an open invitation to comment on the discussion paper describing a technical review of the Gene Technology Regulations (2001) with focus on options for regulating new technologies, the Veterinary Manufacturers and Distributors Association (VMDA) is pleased to provide opinion and in this regard, we draw from the extensive commercial experience of our membership with a range of live viral, bacterial, mycoplasmal and coccidial agents, as well as their knowledge of new generation vaccine antigens which are in development.

In summary, VMDA endorses the principle that organisms produced using methods that create single nucleotide substitutions or deletions, are genetically indistinguishable from, and do not pose a greater risk than, organisms which could have occurred naturally or have been created by metagenesis techniques, currently excluded from regulation.

We therefore support Option 4, excluding oligo-directed metagenesis and site-directed nuclease techniques, SDN-1 and SDN-2, from regulation, on the basis of the outcomes they produce.

Further, we propose that any technique that results in the substitution of nucleotide base pairs, or the deletion or duplication of nucleotide sequences, should also be excluded from regulation, as these will similarly result in organisms that are genetically indistinguishable from, and do not pose a greater risk than, organisms that could have occurred naturally or have been created by mutagenesis techniques that are currently excluded from regulation.

In all of these cases, no novel gene sequences are introduced that may confer novel properties that are beyond the evolutionary potential of the organism or population. The evolution potential is the range of genome features and resulting phenotype that are accessible by mutational processes, whether occurring at a natural rate or at an increased rate due to random mutagenesis, directed mutagenesis or artificial selection.

The Discussion Paper: *Options for regulating new technologies*, October 2016, suggests that an unwanted consequence of applying Option 4 to plants and possibly animals, would be that it would be difficult to avoid extending the same exclusion to disease causing micro-organisms in which “small sequence changes might give rise to significant risks.” This statement is at odds with the principle outlined above, that organisms that are genetically indistinguishable from, and do not pose a greater risk than natural or non-GMO organisms, should not attract a greater regulatory burden. Rather this suggests that the risks associated with directed mutagenesis of pathogenic micro-organisms using gene technology are greater than the risks associated with random mutagenesis and/or directed selection.



It is not clear why directed mutagenesis should pose higher risks than random mutagenesis, with or without selection for novel traits. The risks associated with genetic manipulation of pathogenic micro-organisms can include increased disease burden due to increased pathogenicity, altered tissue tropism, extended host range, and resistance to existing treatment methods, or direct harm due to toxicity or allergenicity of proteins and metabolites produced by the organism. In the absence of novel gene sequences that may alter these traits however, these risks may only be realised as the result of nucleotide substitutions, small indels which may truncate or extend open reading frames, and deletions or duplications of entire genes. These risks are not unique to the use of gene technology to effect such genetic changes, and apply equally to organisms modified using methods that are not considered to be gene technology, such as repeated passage *in vitro*.

There is a long history of the purposeful and successful use of *in vitro* passage to alter the genome and change the phenotype of micro-organisms. The history of safe use of these 'natural' (contrasting with 'unnatural' gene technology) means to modify the genomes of pathogenic micro-organisms, without additional regulation beyond that appropriate to the 'risk group' of the organism, suggests that such modification of pathogenic micro-organisms does not pose any additional risk. The use of gene technology to achieve the same range of genetic modifications does not therefore, pose a level of risk that justifies regulation under the Gene Technology Act.

#### **Genetic modifications achieved by non-GM means.**

Attenuated vaccine strains of pathogenic micro-organisms are frequently produced by extended *in vitro* passage in artificial media or on cultured cells. Typically, for clonal organisms such as bacteria and viruses, this repeated passage in an artificial environment results in the accumulation of random mutations. These mutations include nucleotide substitutions, small indels which may truncate or extend open reading frames, and deletions or duplications of entire genes. Specific examples are listed in Table 1 (ATTACHMENT 1).

In Table 1 are compiled a number of published accounts of the attenuation of vaccine strains by *in vitro* passage and evolutionary studies examining the effect of selection during *in vitro* passage. In this regard, we have sought to highlight examples where the genetic changes involved have been described, to note the rare cases where the result is not attenuation of virulence, and to consider the broad range of organisms that may be manipulated in this manner.

As vaccination is the only effective treatment available for many viral infections for instance, there are many accounts of *in vitro* passage to effect genetic and phenotypic changes. There are fewer accounts where the genetic changes responsible for changes in virulence have been investigated, as the use of gene technology to effect changes has become more common as full viral genomic sequences have become easier to generate. Where sequencing of such organisms has been performed, genetic changes have ranged from a number of nucleotide substitutions (Tsugawa and Tsutsumi 2016, Grebennikova *et al* 2004, Krug *et al* 2015, Varela *et al* 2016), to minor insertions and frameshift mutations (Krug *et al* 2015, Spatz *et al* 2012), to large scale deletions involving the removal of multiple open reading frames (Spatz *et al* 2012, Krug *et al* 2015), with the possible creation and expression of fusion proteins (Karron *et al* 1997).

Genome changes during repeated rounds of growth on artificial growth media have been investigated in studies designed to assess the ability of bacteria to respond to selection through *de novo* mutation. Repeated passage of bacteria has been demonstrated to result in nucleotide substitutions (Gonzalez-Carrillo *et al* 2016, Kubicek-Sutherland *et al* 2016), frameshift mutations (Kubicek-Sutherland *et al* 2016),



large deletions removing multiple open reading frames (Gonzalez-Carrillo et al 2016, Nilsson *et al* 2005) and duplications (Gonzalez-Carrillo *et al* 2016, Domenech *et al* 2014).

Eukaryotic pathogens have also been modified by *in-vitro* passage, usually where there exists a clonal strain that has lost the ability to reproduce sexually and re-gain genetic functions. The complexity of the eukaryotic genome limits our ability to identify genetic changes due to *in vitro* passage, however one study describes mutations occurring during passage of a clonal strain of the fungi, *Candida glabrata*, an opportunistic human pathogen, on a murine macrophage cell line. Whole genome sequencing identified nine nucleotide substitutions, of which three resulted in non-synonymous changes in gene coding regions (Brunke *et al* 2014).

These examples demonstrate that the full range of genetic changes, absent the introduction of novel nucleotide sequences from another organism, may be obtained by extended passage of micro-organisms under artificial conditions. Single nucleotide substitutions, gene deletions and sequence duplications occur in response to selection for growth under artificial conditions or due to the absence of selection maintaining gene sequences that are advantageous *in vivo* but not required for growth *in vitro*.

#### **Phenotypic modifications achieved by non-GM methods.**

In most of the cases cited above, repeated passage was performed with the intention of reducing the virulence of a pathogenic organism with respect to the main host.

Porcine Reproductive and Respiratory Syndrome (PRRS) virus was attenuated as the result of 50 single nucleotide substitutions and one codon substitution (Grebennikova *et al* 2004). African Swine Fever (ASF) virus was attenuated as a result of seven single nucleotide substitutions, a frameshift mutation in a predicted ORF, and two deletions of 6bp and 749bp (Krug *et al* 2015). Respiratory Syncytial Virus (RSV) was attenuated following *in vitro* passage at low temperature due to seven single nucleotide substitutions and a large deletion, eliminating expression of two genes.

However an exception has been reported by Varela *et al* 2013, who passaged Schmallenberg virus some 32 times in a sheep cell line and assessed the effect on neurovirulence in a mouse model. Unexpectedly, they detected an increase in neurovirulence, attributed in a later paper (Varela *et al* 2016) to six single nucleotide nonsynonymous transition substitutions in the Gc glycoprotein which occurred during repeated *in vitro* passage. Note that in this study, the initial passage was generated from an infectious clone, which would be classed as 'gene technology' under the current legislation, however the subsequent viral passages in which the mutations occurred were clearly not gene technology. This example demonstrates that increase in virulence associated with increased replication and enhanced cell to cell spread may occur due to mutations accumulating during repeated viral replication *in vitro* on cultured cells.

In bacteria, the expected attenuation in virulence was observed following *in vitro* passage of *Nocardia brasiliensis*, due to the accumulation of 36 single nucleotide substitutions and 17 deletions, affecting 213 genes (Gonzalez-Carrillo *et al* 2016). Similarly, passage *in vitro* led to attenuation of *Mycobacterium tuberculosis* in mice, attributed to a large duplication encompassing approximately 300 genes (Domenech *et al* 2014).

However increased pathogenicity, in the form of resistance to treatment, was observed by Kubicek-Sutherland et al 2016 following *in vitro* passage of Methicillin-resistant *Staphylococcus aureus*. In this case, the aim was not to attenuate but rather to assess the ability of the organism to adapt to the presence of antimicrobial peptides. *In vitro* passage in the presence of animal or plant antimicrobial peptides induced resistance to the range of antimicrobial peptides as well as several antibiotics, apparently due to 1-3 single



nucleotide substitutions in known regulatory and virulence genes. This example of increased virulence following *in vitro* passage is not unique. The insect pathogen, *Xenorhabdus nematophila*, was found to have increased in virulence following *in vitro* passage in the absence of any selection for altered traits (Chapuis et al 2011). Unfortunately in this case, the genetic basis of the increase in virulence is not known.

In the one example of characterised genetic changes occurring during *in vitro* passage of a eukaryotic pathogen, the resulting phenotypic changes included increased virulence and altered tissue tropism in a mouse model of infection (Brunke *et al* 2014).

These examples demonstrate that the risks inherent in the modification of the genomes of micro-organisms using methods that are not classed as 'gene technology' include increased pathogenicity, altered tissue tropism, and resistance to existing treatment methods.

## Discussion

It is thereby amply demonstrated from the examples listed in Table 1 that *in vitro* passage of pathogenic micro-organisms can result in organisms harbouring gene deletions, gene knockout through frameshift mutations and insertions, and single nucleotide substitutions. The phenotypic consequences of these genetic modifications are most often attenuation of virulence in the natural host, but may in rare cases include increased virulence and changes in tissue tropism in animal models of disease, or potential resistance to current methods of treatment.

Despite this demonstrated risk, *in-vitro* passage to create attenuated strains of pathogenic micro-organisms has a history of mainly safe use, beginning in 1921 with the production of the Bacillus Calmette-Guérin (BCG) strain of *Mycobacterium bovis* (see Gonzalez-Carrillo et al 2016, note Plotkin 2005 claims 1927). This might be because changes in risk due to gene deletions, duplications and nucleotide substitutions are moderate relative to the existing degree of pathogenicity, and because pathogenic micro-organisms are already subject to containment and work practices commensurate with their 'risk group' classification in AS/NZS 2243.3 and similar standards worldwide. Furthermore, where the ultimate aim is to create an attenuated vaccine strain that will be released from containment into the environment, extensive safety and effectiveness studies are performed in containment to demonstrate the stability and usefulness of attenuation prior to any use in the field

There is no reason to believe that directed nucleotide substitution or deletion of genes from the genomes of micro-organisms would pose a greater risk than that posed by similar genetic modifications that occur during 'traditional' *in vitro* passage. While it is true that a level of risk does exist, VMDA contends that this risk is adequately managed without the additional regulatory burden imposed by the Gene Technology legislation. There is every reason that micro-organisms should be included in and governed by the principle that organisms that are genetically indistinguishable from, and do not pose a greater risk than natural or non-GMO organisms, and should not attract greater regulatory burden. Hence, in supporting Option 4, which excludes oligo-directed mutagenesis, SDN-1 and SDN-2 from regulation, VMDA also proposes the clarification of Schedule 1 Item 1 to additionally exclude other techniques that result in single nucleotide substitutions and gene deletions in all organisms, including micro-organisms.



## Recomendations

*Consultation Question 1: Which option/s do you support, and why?*

VMDA supports Option 4, excluding oligo-directed mutagenesis, SDN-1 and SDN-2, from regulation on the principle that organisms produced using methods that create single nucleotide substitutions or deletions are genetically indistinguishable from, and do not pose a greater risk, than organisms which could have occurred naturally or have been created by mutagenesis techniques which are currently excluded from regulation on the basis of a long history of safe and effective use.

*Consultation Question 2: Are there other risks and benefits of each option that are not identified in this document?*

None identified

*Consultation Question 3: Is there any scientific evidence that any of options 2-4 would result in a level of regulation not commensurate with risks posed by gene technology?*

The evidence presented above demonstrates that there is no reason to exclude micro-organisms from Option 4.

*Consultation Question 4: How might options 2-4 change the regulatory burden on you from the gene technology regulatory scheme?*

Option 4 would significantly reduce the regulatory burden on the core business of VMDA members if extended to include other techniques that generate the same outcomes (single nucleotide substitutions or deletions). The 'capture' of these techniques within the current regulations greatly inhibits the ability of our members (at least) to recruit participants for field trials, where there is consequential uncertainty around what additional licence conditions and compliance obligations might be required (additional those applied by the Australian Pesticides & Veterinary Medicines Authority, APVMA, who would also review applications for potential field use).

*Consultation Question 5: How do you use item 1 of Schedule 1, and would it impact you if this item was changed?*

Schedule 1 Item 1 is used by us to guide the rational design of candidate vaccine organisms, with the aim of optimising the cost-effective generation of potential vaccine strains for both local and global need while minimising the ongoing regulatory burden. Explicitly excluding deletion mutants, oligo and single-directed nucleotide mutants, would greatly enhance the generation and subsequent clinical evaluation of potential new vaccines.

*Consultation Question 6: Might contained laboratory research on GM gene drive organisms pose different risks to other contained research with GMOs, and how could these risks be managed? Supporting information and science-based arguments should be provided where possible.*

No comment is offered.

*Consultation Question 7: What RNA interference techniques are you using, and are there RNA interference techniques that you believe have unclear regulatory status? Please provide details of the techniques and science-based arguments for whether these techniques pose risks to human health or the environment.*

No comment is offered.



*Consultation Question 8: Do you have proposals for amendments to any other technical or scientific aspects of the GT Regulations? All proposals should be supported by a rationale and a science-based argument.*

The arguments presented above aim to support clarification of Schedule 1 Item 1, to exclude from regulation, gene deletion and single nucleotide mutants that do not encode novel nucleotide sequences. VMDA presents that this would simplify the adoption of Option 4, and would be commensurate with the risk posed by such organisms.

Second and whilst not the primary aim of this review, VMDA perceives an invitation to comment on the process of requesting and receiving a Permit to conduct field studies involving a technology within the jurisdiction of the OGTR. To this end, we understand the requirement to nominate potential trial co-operators so that OGTR can consult with relevant agencies and maintain open communication with interested parties in a geographical area where a new technology might be tested, but this can present extreme practical difficulties.

When a commercial enterprise is approached to participate in a clinical or field evaluation, the first things that they request are copy of the proposed investigation protocol and information on any things that may affect their normal commercial practice, such as but not limited to, changed management techniques, pre-marketing withholding periods, additional quarantine controls and signage, and public exposure. Agreement to participate in the evaluation or not, will likely depend on the answer to these questions.

In dealing with field trial Permit applications to the Australian Pesticides and Veterinary Medicines Authority (APVMA), there is provision for a draft Permit to be provided in advance of finalist trial candidates being nominated to APVMA, wherein any constraints on a given farm are known and the farm formally agrees or not, to participate, before a study is commenced.

According to OGTR process however, a potential trial candidate must be identified, described and nominally agreed, perhaps 6 months before the Permit is granted and any of the constraints described above are known, at which time the candidate may decline, and the process starts again. There is an absolute need for a solution which allows preliminary agreement with OGTR as to what constraints might be imposed on Permit-controlled study(ies), therein allowing informed potential trial candidates to nominate, which then facilitates the OGTR consideration and communication process in a more targeted way, with expedience.

VMDA believes that we can assist the Regulator to find a solution to this problem which otherwise encourages new technology progression and R&D investment to firstly be made, outside of Australia.

Yours sincerely,

Jim Adams  
Executive Director  
VMDA



**Attachments:**

**Attachment 1: Table 1. Studies demonstrating genetic modifications due to repeated *in vitro* passage.**

**Attachment 2: References**

**Attachment 3: Copies of papers referred to in Attachment 2 (zipped file via email).**



**Table 1. Studies demonstrating genetic modifications due to repeated *in vitro* passage.**

Citation	Organism	Summary	Genetic modification
Brunke S, Seider K, Fischer D, Jacobsen ID, Kasper L, et al. (2014)	Haploid yeast fungal <i>Candida glabrata</i> (Human pathogen)	Maintenance <i>in vitro</i> on a murine macrophage cell line resulted in stable genetic modification, increased virulence and altered organ tropism in mice	Nine SNPs, four non-coding, five in coding regions, of which three were non-synonymous
Chapuis É, Pagès S, Emelianoff V, Givaudan A, Ferdy J-B (2011)	Xenorhabdus nematophila, a bacterial pathogen of insects transmitted by a nematode vector	Repeated passage (40 cycles) in liquid culture media. Two treatments: low density (high dilution factor between passages) and high density (low dilution factor). Both passaged treatments resulted in increased virulence in insects	Unknown
Domenech, P., Rog, A., Radomski, N., et al.. (2014).	<i>Mycobacterium tuberculosis</i>	A large duplication causing attenuation <i>in vivo</i> and observed in clinical isolates was replicated by selection for growth <i>in vitro</i>	A 350 kbp duplication encompassing 300 genes, flanked by short repeat sequences
Gonzalez-Carrillo, C., Saucedo-Millan, C., Garza-Lozano, H. G., et al. (2016)	<i>Nocardia brasiliensis</i>	When tested in BALB/c mice the 200th <i>in vitro</i> passage was reduced in virulence producing 10% incidence of minor lesions compared with 90% incidence of more severe lesions produced by Passage 0.	The 200 <sup>th</sup> passage demonstrated 36 SNPS (16 non-coding, 5 synonymous and 15 nonsynonymous), and 17 deletions ranging from 8 to 262,913 bp affecting 213 genes. Duplication (×12) of a 9bp motif disrupted a predicted ORF, while a further duplication of 70bp occurred in an intergenic region
Grebennikova, T. V., Clouser, D. F., Vorwald, A. C., Musienko, et al. (2004).	Porcine Reproductive and Respiratory Syndrome Virus	Extended cell culture passage <i>in vitro</i> (249 passages on an african green monkey cell line) resulted in significant attenuation	Fifty single nucleotide changes (thirty non-synonomous), and one in-frame three bp deletion
Karron, R. A., Buonagurio, D. A., Georgiu, A. F., et al. (1997).	Respiratory syncytial virus (RSV) Negative sense ssRNA <i>Pneumoviridae</i>	A candidate vaccine was attenuated by 32 passages in vero cells at a lowered temperature. The resulting candidate vaccine was trialed in adults and children and found to be too attenuated to provide efficacy against RSV	Sequencing demonstrated that the viral genome harboured a deletion that removed all but the first 31 nucleotides of the SH (small hydrophobic) gene and last 60 nucleotides of the G (glycoprotein) genes, potentially creating an SH:G fusion protein



<p>Krug PW, Holinka LG, O'Donnell V, <i>et al.</i> (2015)</p>	<p>African Swine Fever Virus (Enveloped dsDNA 190kb)</p>	<p>A virulent isolate of ASFV was passaged <i>in vitro</i> on Vero cells. Samples taken at passages 30, 60, 80, and 110 were assayed for virulence <i>in vivo</i>, demonstrating decreasing virulence with increased passage number. These isolates were sequenced to identify genetic changes responsible for attenuation</p>	<p>Non-synonymous point mutations increased from three at passage 3 to seven at passage 110, although one mutation at Passage 3 had reverted at 110. In addition one frameshift mutation occurred and a gly codon was inserted into a predicted ORF. Deletions occurred encompassing several Open Reading Frames. A deletion of approximately 6Kb occurred in the left variable region of the genome. A deletion of 749bp occurred in the right variable region of the genome. Additional independent deletions events involving the same regions were observed at intermediate passage levels, however populations of these genome variants were minor or undetectable by passage 110</p>
<p>Kubicek-Sutherland, J. Z., Lofton, H., Vestergaard, M., <i>et al.</i> (2016).</p>	<p>Methicillin-resistant Staphylococcus aureus (MRSA), human clinical isolate</p>	<p><i>In vitro</i> passage in the presence of human, mouse, pig or plant antimicrobial peptides. Seven passages strains were identified that stably acquired resistance to the range of antimicrobial peptides and also several antibiotics within 168-322 generations</p>	<p>Mutant strains contained 1-3 nucleotide substitutions relative to the WT strain in genes including regulatory and recognized virulence genes. All four control passaged strains demonstrated frameshift or substitutions resulting in early stop codons within two-component regulatory genes associated with virulence. This was not observed in selected strains, suggesting selection for maintenance of these genes</p>
<p>Nilsson, A. I., Koskiniemi, S., Eriksson, S., <i>et al.</i> (2005).</p>	<p><i>Salmonella enterica</i></p>	<p>Repeated propagation on solid media by transfer of single colonies up to 270 times.</p>	<p>Deletions were observed ranging from 1200 to 173000bp, and encompassing up to 11 genes This study focused on genome size reductions by spontaneous deletions and did not report substitutions or insertions</p>



<p>Spatz, S. J., Volkening, J. D., Gimeno, I. M., Heidari, M., &amp; Witter, R. L. (2012).</p>	<p>Gallid Herpesvirus-2 (GaHV-2), Marek's disease virus, Alpha herpesvirus</p>	<p>Passage <i>in vitro</i> on Chicken Embryo Fibroblasts, with stocks formed at 11, 31, 41, 61, 81 and 101. Stocks were sequenced and compared with the parent 648A strain (Passage 0)</p>	<p>A 632 bp deletion encompassing the Meg oncogene binding site was detected at passage 41 and was the predominant genotype by passage 101. Two further variant genomes harbouring additional large deletions occurred at late passages and as minor variants at passage 101. One of these (948bp) involved the virulence factor vLipase, the second (820bp) involved the virulence factor vIL8. Insertions of 1 or 2 bp were recorded that truncated or modified various ORFs. Single nucleotide polymorphisms occurred in various passages, however these fluctuated in frequency in successive passages, Only one, a G to A transition in a non-coding promoter region was predominant at passage 101</p>
<p>Varela M, Schnettler E, Caporale M, Murgia C, Barry G, McFarlane M, <i>et al.</i> (2013)</p>	<p>Schmallenberg virus Bunyaviridae enveloped segmented negative sense ssRNA pathogen of ruminants</p>	<p>Infectious clone of SBV was passaged 32 times in the sheep cell line CPT-Tert. (Note that the RNA sequence of the clone was found to be identical to the wild type). The effect of passaging on neurovirulence was assessed <i>in vivo</i> in a mouse model. The passaged virus demonstrated increased virulence in murine brain compared with the parent strain</p>	<p>Determined in the study below</p>
<p>Varela, M., Pinto, R. M., Caporale, <i>et al.</i> (2016).</p>	<p>Schmallenberg virus Bunyaviridae</p>	<p>Non-synonymous substitutions in the Gc glycoprotein gene that occurred during <i>in vivo</i> passage identified as conferring increased neurovirulence in mice</p>	<p>Six single nucleotide nonsynonymous transition substitutions occurred in the Gc glycoprotein. (One transversion was synonymous). Additional substitutions in Ns were found to be attenuating</p>



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