EUROPEAN COMMISSION
HEALTH & CONSUMER PROTECTION DIRECTORATE-GENERAL

Directorate C - Scientific Opinions
C2 - Management of scientific committees; scientific co-operation and networks

COMMENTARY BY THE SCIENTIFIC COMMITTEE ON ANIMAL NUTRITION ON DATA RELATING TO TOXIN PRODUCTION SUBMITTED BY INTERVET INTERNATIONAL GMBH ON ANOTHER PRODUCT (TOYOCERIN® PRODUCED BY ASAHI VET S.A.).

(ADOPTED ON 5 DECEMBER 2001)

1. BACKGROUND

Following the SCAN opinion of 17 February 2000 on the safety of use of *Bacillus* species in animal nutrition, additional data on the ability to produce toxins was requested by the Commission from Companies whose products contained or were derived from strains of *Bacillus* spp.

One company, Intervet International GmbH marketing the product Paciflor® whose active ingredient is a strain of *Bacillus cereus* CIP 5832, submitted data which led SCAN to the Opinion that the strain was capable of producing enterotoxins and so was unsafe for use as an additive. In their submission, Intervet also included comparative data on other commercial products. One product included in this comparison was the feed additive Toyocerin® whose active ingredient also consists of viable spores of a different strain of *B. cereus.*

Toxin production by the Toyocerin® has been separately assessed by SCAN on the basis of data provided by Asahi Vet S.A., the company marketing Toyocerin® in Europe. It was concluded that, although the strain contained gene sequences derived from some elements of *B. cereus* enterotoxins, the entire toxins were not expressed and, in the absence of any evidence of cytotoxicity, the strain was safe for use. However, the data produced by Intervet on Toyocerin® was not consistent with this conclusion and, if examined in isolation, could suggest that the product Toyocerin® carried a similar risk to that posed by Paciflor®.

The apparent discrepancy between the data produced by the two Companies presents two issues. The first relates to the immediate question of the safety of the Toyocerin® product and the second to the more general issue of the wisdom of permitting comparative data to be included in Dossiers when this is not a requirement of the relevant legislation.

2. TERMS OF REFERENCE

The Scientific Committee on Animal Nutrition is requested to consider the data submitted by the Competitor and to advise the Commission whether this would lead to any revision of the conclusions drawn by SCAN on the ability of product Toyocerin® to produce toxin(s).

3. SCAN OPINION

3.1. Data produced by Intervet on toxin production by the Toyocerin® strain

Two commercially available immunological methods designed to detect elements of the most commonly encountered enterotoxins, the hemolysin BL toxin (Hbl, Oxoid kit) and the non-haemolytic toxin (nhe, TECRA kit), gave positive results.

Single primer pairs for the Hbl and Nhe toxins gave amplification products.

A cytotoxity assay using Vero cells and the rate of ATP reduction as the detection method was positive for dilutions of culture supernatant (brain-heart infusion [BHI] medium) as low as 1 in 32.

Cytotoxicity assays using three human cell lines (HCT-8, HUTU, Hep-2) and two animal cell lines (Vero, MDBK) were all positive with 1 in 20 dilutions of supernatents from BHI (a nutrient rich medium) but negative with supernatents produced from rice medium (a nutrient poor medium).

3.2. Source of bacterial culture

Access to cultures of organisms deposited with recognised culture collections may be restricted if the strain in question is commercially sensitive. The alternative of isolating the strain from the product, although straightforward, may inadvertently select for clone with different phenotypic characteristics or even a contaminating strain. For the results produced by Intervet to be truly comparable it would have to be demonstrated that the strain used in their laboratories was genetically indistinguishable from that deposited in the culture collection and used by Asahi Vet for the production of Toyocerin®.

3.3. Immunological assays

SCAN recognised that the two commercially available kits for the detection of enteroxins of *B. cereus* provide a rapid and convenient method for screening for toxin production. However, for a variety of reasons they were not considered definitive and, as their use would always have to be followed by other studies, SCAN considered them optional. These tests were not included in the Dossier prepared by Asahi Vet and so no direct comparisons can be made.

The Oxoid kit is based on an antibody to one component (the L_2 component encoded by *hblC*) of the hemolysin BL toxin. In the data provided by Asahi Vet primer pairs specific to *hblC* failed to generate a PCR product and, in separate study, Western blot analysis using monoclonal antibodies to each of

the three components of the hemolysin BL toxin detected these components only in the supernatant of a positive control strain and not the Toyocerin® strain. Consequently the Toyocerin® strain would not be expected to react positively with the Oxoid kit.

The TECRA kit also detects only one element (NheA) of the tripartite non-haemolytic toxin. Data provided by Asahi Vet showed that PCR primers specific for *nheA* did give rise to a product of the expected size (1228bp) confirming the presence of at least part of the gene encoding the NheA component. Consequently, assuming expression, it is likely that supernatant from the culture of the Toyocerin® strain would test positive with the TECRA kit.

3.4. PCR results

Intervet reports PCR studies using the primer pairs recommended in the SCAN Opinion and intended as a broad detection method for the hemolysin BL and non-haemolytic toxins. The primers are designed to amplify regions that are believed to be highly conserved and thus likely to be essential for biological activity. Each set detects the presence of two of the three structural genes in the operon (*hplD-hblA* for the Hemolysin BL toxin and *nheB-nheC* for the non-haemolytic toxin). Using these primers, amplification products of the expected size were obtained with the Toyocerin® strain.

Asahi Vet opted for a more specific investigation and designed primer pairs for each component of the two tripartite toxins. These demonstrated the presence of fragments derived from *hblA* component of the hemolysin BL toxin but equally conclusively the absence of the genes encoding the other two components (*hblC* and *hblD*). Similarly, the *nheA* gene could be detected but not the genes (*nheB* or *nheC)* coding for the remaining components of the non-haemolytic toxin.

While it might be possible to reconcile the PCR data relating to the hemolysin BL toxin since the results obtained by both Intervet and Asahi Vet indicate the presence of *hblA*, the same is not possible with the non-haemolytic data. Unlike the SCAN recommended primers, which have been used with many hundreds of *B. cereus* strains and shown to be reliable, the primers designed by Asahi Vet have not been extensively used. Consequently there is a possibility that they are directed to more variable regions in the genes and thus prone to false negatives.

3.5. Cell cytotoxicity

Data on cell cytotoxicity provided by Asahi Vet was obtained using concentrated supernatant prepared by filtration through membranes able to retain proteins of >10kDa. Consequently, any low molecular weight material that might confound the assay would have been largely removed. In the case of the Intervet data, all studies were made with culture supernatant as recovered by centrifugation or dilutions of culture supernatant. Both companies used BHI as the growth medium.

Two different clones of the Vero cell line were used in the studies commissioned by Asahi Vet in two contract laboratories. Cytotoxicity was measured using the extent of cleavage of MTT as evidence of reduced metabolic activity and the supernatant used was concentrated five-fold. Appropriate positive and negative control strains were included in the study. No evidence of cytotoxicity was found associated with the Toyocerin® strain, a result consistent with the PCR and antibody studies.

Very different results were reported in the Invervet Dossier using a Vero cell line. Here a 1-in-8 dilution of culture supernatant derived from the culture of the Toyocerin® strain led to a 38% reduction in ATP production and a 1-in-32 dilution to a 26% reduction. Higher concentrations of supernatant (1-in-2) were said to produce visible disruption to the cell layers. Further cell studies were made with a variety of human and animal cell lines. These used detection methods that ranged from microscopic observation and the use of specific fluorescent stains to distinguish between vital and membrane damaged cells, to quantitation by measuring the retention by cells of the stain crystal violet. All gave results using supernatant from the Toyocerin® strain grown in BHI similar to the positive control and suggested a toxic presence. However, no negative control was included and these later studies lack the discrimination of the Vero cell studies that included a quantitative measure of metabolic status.

4. CONCLUSIONS

SCAN concludes that:

- In the absence of evidence that confirms the identity of the culture tested as being the Toyocerin® strain, data produced by Intervet should be treated with some caution.

- The positive finding with the TECRA test kit is consistent with the data produced by Asahi Vet and would be expected.

- The positive finding with the Oxoid kit is unexpected given the data produced by Asahi Vet on absence of the gene encoding the L_2 component of the toxin and the gene product itself. However, the ELISA kits are known to give rise to false positives and their use is not considered definitive.

- The PCR primers recommended by SCAN for the detection of the hemolysin BL and the non-haemolytic toxins have been extensively tested and found to give results which are robust and reproducible. Obtaining amplification products of the expected size with these primer sets casts doubts on the negative results obtained with the far less extensively tested primers used by Asahi Vet

- It is not possible to explain with any confidence the differences in the cytotoxicity studies made by the two Companies. However, it should be noted that Vero cells are subject to clonal variation and can vary considerably in their response to trophic factors and other components introduced into the media. Thus the origin of the cells, passage number and many environmental factors can influence the results obtained. Of the two sets of studies SCAN favours the data

produced by Asahi Vet which recognised this potential for variation, obtained cells from two sources known to differ, performed the work in two different laboratories and used one of the detection systems for estimating metabolic status recommended by SCAN.

The results presented by Asahi Vet in series of Dossiers are internally consistent and allow a conclusion on the safety of the product to be reached. PCR studies are included which failed to detect four of the six components of the two principle toxins. Since it is known that all three components of the hemolysin BL toxin need to be present for full toxicity and that in all probability the same is true for the non-haemolytic toxin, no evidence of cytotoxicity would be expected. Use of antibody probes for the expressed proteins and the lack of cytotoxicity in *in vitro* and *in vivo* provided the evidence to back this expectation.

The PCR data provided by Intervet does bring into question a key element of the Toyocerin® results and the conclusions that might be drawn and consequently cannot be ignored. However, since the Intervet data also is open to question, SCAN is unwilling to revisit its opinion on this aspect of the safety of the Toyocerin® product[1] until more definitive data can be provided.

5. RECOMMENDATIONS

SCAN recommends the use of an independent third party given access to the deposited strains of both the Intervet and Asahi Vet products to resolve this issue. This laboratory should repeat the PCR studies using the general and specific primer sets for the hemolysin BL and non-haemolytic toxins and the sub-elements of these toxins using both strains and the two operons should also be fully sequenced in both strains.

This case illustrates the difficulty for the Companies and for the Commission following the inclusion of data on competitor products in a Dossier and the potential for abuse that could arise. SCAN recommends that the Commission should take action to avoid this situation occurring in the future by discouraging inclusion of data on the safety of competitor products in dossier unless required by the relevant legislation. If there are legitimate reasons for product comparisons, these should be done by an independent laboratory and the results made available to all parties involved.

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[1] Report of the Scientific Committee on Animal Nutrition on product Toyocerin® for use as feed additive, adopted on 5 December 2001.
Available at: http://europa.eu.int/comm/food/fs/sc/scan/outcome_en.html