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Community Reference Laboratory for Feed Additives Authorisation

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CRL Evaluation Report on the Analytical Methods submitted in connection with Section II, 2.5 (Control Methods) of the Application for Authorisation as a Feed Additive according to Regulation (EC) No 1831/2003

Dossier related to:	EFSA-Q-2005-167
Name of Additive:	ColiCure® for horses
Active Agent(s):	*Escherichia coli* E-101-88, LMG S-1714
Rapporteur Laboratory:	Community Reference Laboratory for Feed Additive Authorisation, IRMM, Geel, Belgium (CRL-FAA)
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Date:	20/09/2006

EXECUTIVE SUMMARY

In the current application authorisation is sought for the microbial feed additive ColiCure® under the category ‘zootechnical additives’, functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. The active agent in the additive is *Escherichia coli* E-101-88, LMG S-1746. Specifically, authorisation is sought to use ColiCure® for horses. It is proposed that one bottle of 100 ml ColiCure® which contains at least 1 x 10^9 colony forming units (c.f.u.)/ml of the active agent *Escherichia coli* E-101-88, LMG S-1746 suspended in phosphate buffered saline is added to a small amount of feedingstuffs for horses for immediate consumption. For the quantification of the active agent (*Escherichia coli* E-101-88, LMG S-1746) of ColiCure® in the feed additive and feedingstuffs, an appropriate non-selective surface plate count method based on well-known principles using nutrient agar supplemented with 5 % bovine blood and an incubation temperature of 37 °C was proposed by the applicant. The method is considered suitable for the intended purpose. For official controls of the *feed additive* ColiCure® and if required of *feedingstuffs* supplemented with ColiCure® corresponding officially recognised standard methods such as ISO and/or CEN methods for example ISO 4832, ISO 16649-2 or ISO 21528-2 are recommended. The identity of the microbial strain was analysed by a range of techniques including microscopy, serology, biochemistry, polymerase chain reaction (PCR) and restriction enzyme analysis using three enzymes. The applicant used amplified fragment length polymorphism (AFLP) and pulsed field gel electrophoresis (PFGE). PFGE is a generally recognised standard methodology for microbial identification and is considered suitable for official controls.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

KEYWORDS

ColiCure®, feed additive, *Escherichia coli*, zootechnical additive, horses

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BACKGROUND

ColiCure® is a feed additive for which authorisation is sought under the category 'zootechnical additives', functional group 'gut flora stabilisers' according to Annex I of Regulation (EC) No 1831/2003. ColiCure® is a suspension of a purified and stabilized *Escherichia coli* strain originally isolated from the intestine of a healthy horse [1]. According to the applicant, the feed additive contains at least 1 x 10^9 colony forming units (c.f.u.) of *Escherichia coli* per milliliter (ml) suspended in phosphate buffered saline at pH 7.0 [1]. The bacterial suspension is intended to be given in a small amount of feed to horses [1]. The strain of *Escherichia coli* used as active agent, *Escherichia coli* E-101-88, LMG S-1746 is deposited at the 'LMG Culture Collection' University of Gent, Belgium [2]. The intended use of the current application (EFSA-Q-2005-167) is for horses of any age [1].

TERMS OF REFERENCE

In accordance with Article 5 of Regulation (EC) No 378/2005 on detailed rules for the implementation of Regulation (EC) No 1831/2003 of the European Parliament and of the Council as regards the duties and tasks of the Community Reference Laboratory concerning applications for authorisations of feed additives, the CRL is requested to submit a full evaluation report to the European Food Safety Authority (EFSA) for each application. For this

particular dossier, the methods of analysis submitted in connection with the ColiCure® dossier (EFSA-Q-2005-167) and their suitability to be used for official controls, were evaluated.

EVALUATION

The numbering system under this point refers to the 'Guidelines for the assessment of additives in feedingstuffs, part II: Enzymes and Micro-organisms' (2.5 Control methods), in the following referred to as 'the Guidelines'.

Description of the some of the methods listed under item 2.5.1. of the Guidelines

Qualitative and quantitative composition of the additive

The concentration of the active agent, *Escherichia coli* E-101-88, LMG S-1714 in the additive is at least 1 x 10^9 c.f.u./ml [3]. The method for quantifying the active agent as proposed by the applicant represents a surface plate count method using nutrient agar supplemented with 5 % bovine blood. As the additive is present in high viable numbers the use of a non-selective agar is considered appropriate. The results are reported as colony forming units (c.f.u.) per ml additive. The applicant provided a short method description for plate counting. Serial dilutions of the feed additive are carried out in saline (10^{-1} to 10^{-9}). The agar plates are inoculated with 0.1 ml of each dilution and subsequently incubated at 37 °C overnight (18-24h) [4, 5]. The method is suitable for the intended purpose and might be suitable for official controls in the frame of the authorisation, however the provided method protocol lacked in part information that is usually provided in standard methods. For official controls officially recognised methods such as ISO and/or CEN methods for example ISO 4832, ISO 16649-2 or ISO 21528-2 are therefore recommended.

The production strain was biochemically characterised by carbohydrate fermentation patterns by use of API 20E and API 50CHE and the BIOLOG GN2 microtiter plate design to test the ability of the organism to utilise 95 carbon sources [6]. The results from the biochemical characterisation by carbohydrate fermentation were supplied by the applicant [7].

Serotyping of somatic O-antigen, flagellar or H-antigens and capsular or K-antigens was performed at the Statens Serumsinstitut (Copenhagen, Denmark) and O-antigen typing was also performed at the National Veterinary Institute (Uppsala, Sweden) [7, 8].

The strain was further characterized by a PCR based whole genome fingerprinting technique via selective amplification of restriction enzyme fragments and amplified fragment length restriction polymorphism (AFLP). Four primer combinations were used. The PCR products were separated on agarose gels and visualized by autoradiography. The electrophoretic patterns were scanned and numerically analysed with a computer programme [7]. The applicant provided the pattern of the AFLP fingerprint of the strain [6].

The applicant used in addition pulsed field gel electrophoresis (PFGE) to identify the strain using three different restriction enzymes [7].

The genetic identity of the strain is examined by a combination of techniques. PFGE is considered as a suitable method for official control purposes.

Qualitative and quantitative composition of any impurities in the additive

The applicant analyses the feed additive for microbiological contaminants by plating appropriate dilutions on lactose agar and Columbia agar to obtain single colonies for control of contamination of other bacterial species then *E. coli*. Possible presence of yeasts and moulds is investigated by plating appropriate dilutions on Sabouraud agar plates [9]. Microscopy and an inspection of agar colony morphology are carried out at each production [10]. The methods are not provided in form of complete protocols however the proposed media are considered suitable for the intended purpose. For official controls the corresponding CEN and/or ISO methods, for example ISO 7954 are recommended.

For the analysis of heavy metals, the applicant contracted the National Veterinary Institute (Uppsala, Sweden) to analyse the additive for cadmium (Cd), lead (Pb), arsenic (as) and mercury (Hg) using inductively coupled plasma-atomic emission spectroscopy (ICP-AES), after wet digestion [11]. For official controls various standard methods based on the same analytical techniques and routinely applied by official control authorities are available and recommended by the CRL.

Description of qualitative and quantitative methods for routine control of the active agent in premixtures and feedingstuffs (cf. requirements of Guidelines section 2.5.2)

For the enumeration of the active agent *Escherichia coli* E-101-88, LMG S-1714 in feedingstuffs, the applicant proposes the same surface plating method as for the feed additive [4, 12].

The applicant specifies that the individual horse is given an exact amount of ColiCure® (100 ml) mixed in a small amount of feedingstuffs and not more than is consumed instantly [13]. It is therefore not the intention to incorporate the additive into feedingstuffs which would then be offered for sale commercially. Consequently, the requirement for a method to determine the active substance in feedingstuffs does not seem particularly relevant for official controls.

The applicant provided a standard operation procedure and validation data for the enumeration of the additive in feedingstuffs. According to the information provided, the viable counts of ColiCure® were not affected by the horse feed matrix (Betfor®) and were present in high viable numbers in the concentration range of the feed additive [14]. The use of a non-selective nutrient agar as proposed by the applicant is considered appropriate as the viable counts of the active agent are at such a high concentration at which inherent microbial contaminations in the feedingstuffs would not be expected. The method can be considered appropriate for the proposed purpose. However, for official controls in the frame of this authorisation, corresponding officially recognised Standard methods such as ISO and/or CEN methods, for example ISO 4832, ISO 16649-2 or ISO 21528-2 are recommended.

CONCLUSIONS AND RECOMMENDATIONS

The applicant uses a broad range of conventional methods to enumerate and to characterise the active agent in the additive and to test for the presence of potential impurities in the product. These are considered appropriate for the purpose, however, the CRL recommends for official controls corresponding ISO and/or CEN methods, for example ISO 4832, ISO 16649-2 or ISO 21528-2. A method for the enumeration of the active agent in feedingstuffs is of minor relevance for this authorisation as the additive is added prior to an instant consumption of the feedingstuffs and supplemented feedingstuffs are not offered as independent commercial products.

For the analysis of the identity of the bacterial strain, *Escherichia coli* E-101-88, LMG S-1746, the applicant uses also a range of techniques which are appropriate to identify the strain. As PFGE is already widely used by reference laboratories to identify bacterial isolates it is recommended as a suitable methodology in this context for official controls.

On the basis of the supplied documentation, no supplementary experimental work (testing or method validation) is required.

Recommended text for the register entry, fourth column (Composition, chemical formula, description, analytical method)

Analytical method suitable for official controls: Enumeration surface plate count method using a non-selective or a selective agar (e.g. ISO 4832, ISO 16649-2 or ISO 21528-2). Other methods could also be considered, providing their performance characteristics are at least comparable to the proposed method.

DOCUMENTATION AND SAMPLES PROVIDED TO CRL

The dossier has been made available to the CRL by EFSA. In accordance with the requirements of Regulation (EC) No 1831/2003, samples of the additive ColiCure® for horses have been sent to the Community Reference Laboratory for Feed Additives Authorisation.

REFERENCES

[1] EFSA, Annex III Proposal of Register entry

[2] Technical dossier Section II. Identity, characterisation and conditions of use of ColiCure®. 2.2.2 Biological origin.

[3] Technical dossier, Section II. 2.1.3 Qualitative and quantitative composition

[4] Technical dossier, Section II. 2.5 Control methods

[5] Enclosure II 7.15 SOP for plate counting

[6] Enclosure II 7.4 Results from biochemical characterisation by carbohydrate fermentation and by a PCR based genome fingerprinting technique (AFLP) of the E. coli E101-88

[7] Technical dossier, Section II. 2.2.2 Biological origin

[8] Enclosures II 7.2 Result from serotyping of somatic antigen of the *E. coli* E-101-88; II 7.3 Result from serotyping of somatic antigen of the *E. coli* E-101-88 at the National Veterinary Institute

[9] Technical dossier, Section II. 2.1.4 Qualitative and quantitative composition of any impurities; Enclosure II 7.10 SOP and result from purity test concerning the final product

[10] Technical dossier, Monograph, Section V. 5.4 Control methods

[11] Enclosure II 7.11 Result from testing for heavy metal in the final product

[12] Technical dossier, Section II. Identity, characterisation and conditions of use of ColiCure®. Methods of control. 2.5 Control methods

[13] Technical dossier, Section II. 1.2.4 Conditions of use of ColiCure®

[14] Enclosure 11.5.7 B-10 'Quantitative method for enumeration of live *E. coli* cells per ml in horse feedstuff'

ISO 7954:1987 Microbiology – General guidance for enumeration of yeast and moulds – Colony count technique at 25 °C

ISO 16649-2:2001 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* – Part 2: Colony-count technique at 44 °C using 5-bromo-4-chloro-3-indolyl beta-D-glucuronide

ISO 21528-2:2004 Microbiology of food and animal feeding stuffs – Horizontal method for the detection and enumeration of *Enterobacteriaceae* – Part 2: Colony count technique

ISO 4832:2006 Microbiology of food and animal feeding stuffs – Horizontal method for the enumeration of coliforms – Colony count technique

RAPPORTEUR LABORATORY

The Rapporteur Laboratory for this evaluation was the Community Reference Laboratory for Feed Additive Authorisation (CRL-FAA), Geel, Belgium