Global detection and identification of *Campylobacter fetus* subsp. *venerealis*

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Submitted for publication: 4 July 2005
Accepted for publication: 7 July 2005

**Summary**

Bovine genital campylobacteriosis caused by *Campylobacter fetus* subsp. *venerealis* (Cfv) is a genital infection that threatens the cattle industry. Detection and identification of Cfv are key factors in control programmes. Trade regulations should be based on scientifically and internationally accepted methods of detection and identification of Cfv. Such methods are described in the World Organisation for Animal Health (OIE) *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. A study was conducted to determine which methods are in use in OIE Member Countries and to get an overview of new or improved tests. A questionnaire was sent to OIE Member Countries, and 26 out of 166 were returned. Globally, a diversity of methods for the detection and identification of Cfv are in use. The authors conclude that there is a lack of harmonisation that may have consequences for the description of the health status of countries and may lead to disputes with respect to trade regulations.

**Keywords**


**Introduction**

Bovine genital campylobacteriosis (BGC) is a bacterial venereal disease, which may lead to infertility, early embryonic mortality and abortion (2, 9, 16). The disease is caused by *Campylobacter fetus* subspecies *venerealis* (Cfv) (19). Bovine genital campylobacteriosis has a worldwide distribution (3) and causes high economic losses to the bovine industry. The disease was formerly classified as a List B disease by the World Animal Health Organisation (OIE). It is also known as bovine venereal campylobacteriosis because of its route of transmission (16). As the bacterium is strongly host-restricted, the introduction of artificial insemination (AI) in combination with veterinary health control programmes has reduced the incidence of BGC in many industrialised countries. In developing countries where AI is less common and appropriate animal health surveillance programmes are not optimal, BGC remains a major problem for the cattle industry. According to the OIE *Terrestrial Animal Health Code* (the Code) (10), OIE Member Countries are obligated to monitor the prevalence of BGC in their countries and report the findings to the OIE. These findings are publicly available (http://www.oie.int).

Proper detection and identification of Cfv are key factors in control and eradication programmes. Trade regulations should be based on internationally agreed scientific
methods for isolation and identification of Cfv. The OIE provides these methods in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (the Manual). The Manual describes internationally agreed laboratory methods for disease diagnosis and requirements for the production and control of biological products, while the Code describes health measures to be used by the veterinary authorities of importing and exporting countries to avoid the transfer of agents that are pathogenic for animals or humans, while avoiding unjustified sanitary barriers. The Code and the Manual are key documents in the regulation of trade in live animals and biological products. Their objective is to harmonise the diagnosis and control of animal diseases. The isolation and identification of Campylobacter fetus described in the Manual.

In addition to isolation and identification, subspecies differentiation of the species is important. Cfv is considered to be restricted to the bovine genital tract, but is very closely related to the less host-restricted C. fetus subsp. fetus (Cff) (2). Cff has been isolated from a variety of sources, including ungulates, fowl, reptiles and humans (2). Hosts can be intestinal carriers for long periods and the genital tract can become infected through this persistence. Therefore, Cff may also be isolated from samples that are screened for Cfv. Depending on the statutory import requirements of a specific country or region, bovine semen, embryos or live animals should be free from either Cfv or both Cfv and Cff. It is thus important to have a reliable method for subspecies differentiation and this is also described in the OIE Manual.

In 2002, the authors compiled a questionnaire on the isolation, identification and subspecies differentiation of C. fetus to determine which methods are in use in OIE Member Countries and to get an overview of new or improved tests, as these may lead to an adaptation of the protocols described in the OIE Manual. This paper describes the findings.

Questionnaire

An English-language questionnaire was compiled (Table I) that focused on the isolation and identification of C. fetus based on the methods described in the fourth edition of the Manual, which was published in 2000 (8). Most questions (n = 41) required a simple ‘yes’ or ‘no’ answer and some required a detailed response. Respondents were encouraged to make additional remarks or comments by filling out the reserved fields. In March 2003, the questionnaire was sent to all the Delegates of the then 166 Member Countries of the OIE, who were asked to forward it to the appropriate laboratory experts. Questionnaires were returned to the OIE by post, fax or e-mail within a fixed time frame.

Although a limited number of the questionnaires were returned (26/166 = 16%), the responses represented different global geographical regions. Countries that returned the questionnaire were, in alphabetical order: Australia, Canada, Cyprus, Denmark, the Dominican Republic, Egypt, Germany, Ireland, Israel, Japan, Kuwait, Lithuania, Mozambique, Myanmar, the Netherlands, Norway, Poland, Slovakia, Slovenia, Sweden, Switzerland, Thailand, Ukraine, the United Kingdom (UK), the United States of America (USA) and Zimbabwe (Fig. 1). The responses received to the questionnaire are summarised in Table I and discussed below. Questions that were answered positively are indicated in percentages, but answers that required clarification and remarks are not shown in the Table.

Results

Diagnosis of Campylobacter fetus infections

Reference (as described in the OIE Manual)

The bacteria can be detected by either isolation of the bacteria from the sample, immunofluorescence of the bacteria in the sample, or demonstration of serum or vaginal mucus antibodies against the bacteria in the host.

Results

Globally, all methods are used, however the occurrence and frequency of use of each method strongly differ between countries. Isolation of the bacteria is by far the most used method. None of the respondents perform the immunofluorescence test (IFT) as a stand-alone test, but about one-third of the laboratories uses IFT in combination with isolation.

Collection of samples

Reference

A variety of specimen types have been described (male: preputial mucus or secretions, and semen; female: vaginal mucus, cervicovaginal mucus, aborted foetuses, placentas).

Results

All types of described specimens are sampled, but to a different extent among the countries (Table I). However, there is a marked preference for certain types of specimens in most countries: 96% use preputial samples and 92% use the internal organs of aborted foetuses.
Table I
Summary of the questionnaire on bovine genital campylobacteriosis
A summary of the questionnaire that was sent to World Organisation for Animal Health (OIE) Member Countries and the results (in percentages) for the questions answered positively. The answers to questions that could not be answered by a simple ‘yes’ or ‘no’ have not been included here, but are discussed in the results.

<table>
<thead>
<tr>
<th>Question</th>
<th>Countries responding positively (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>How do you diagnose <em>Campylobacter fetus</em> infections?</strong></td>
<td></td>
</tr>
<tr>
<td>Isolation of the bacterium only</td>
<td>81</td>
</tr>
<tr>
<td>Detection by immunofluorescence only</td>
<td>0</td>
</tr>
<tr>
<td>Immunofluorescence and isolation</td>
<td>33</td>
</tr>
<tr>
<td>Detection of antibodies in vaginal mucus</td>
<td>35</td>
</tr>
<tr>
<td>Detection of antibodies in serum</td>
<td>12</td>
</tr>
<tr>
<td><strong>Collection of samples by:</strong></td>
<td></td>
</tr>
<tr>
<td>Scraping (bull)</td>
<td>15</td>
</tr>
<tr>
<td>Suction (bull)</td>
<td>12</td>
</tr>
<tr>
<td>Preputial washing (bull)</td>
<td>96</td>
</tr>
<tr>
<td>Washing the artificial vagina</td>
<td>42</td>
</tr>
<tr>
<td>Collection of semen</td>
<td>58</td>
</tr>
<tr>
<td>Swabbing vaginal mucus</td>
<td>42</td>
</tr>
<tr>
<td>Suction of vaginal mucus</td>
<td>38</td>
</tr>
<tr>
<td>Washing the vaginal cavity</td>
<td>42</td>
</tr>
<tr>
<td>Collecting internal organs from aborted foetuses/stomach contents</td>
<td>92</td>
</tr>
<tr>
<td><strong>Treatment of samples by:</strong></td>
<td></td>
</tr>
<tr>
<td>Membrane filtration</td>
<td>38</td>
</tr>
<tr>
<td>Liquefying vaginal mucus by cysteine solution treatment</td>
<td>8</td>
</tr>
<tr>
<td>Centrifugation of preputial washings</td>
<td>73</td>
</tr>
<tr>
<td><strong>Use of transport and enrichment medium</strong></td>
<td></td>
</tr>
<tr>
<td>Do you use a transport or enrichment medium?</td>
<td>73</td>
</tr>
<tr>
<td>What medium do you use? (name or protocol and reference if available)</td>
<td></td>
</tr>
<tr>
<td>Is this medium used for enrichment in the laboratory? (Are the samples incubated in this medium when they arrive at the laboratory awaiting further treatment after, for example, two to three days of incubation?)</td>
<td>50</td>
</tr>
<tr>
<td><strong>Isolation media</strong></td>
<td></td>
</tr>
<tr>
<td>What isolation medium (or media) do you use? (name and reference if available)</td>
<td></td>
</tr>
<tr>
<td>Do you prepare the media by combining all separate components or by using commercially available media (powder or prepared)? (please specify the company)</td>
<td>50</td>
</tr>
<tr>
<td><strong>Filtration</strong></td>
<td></td>
</tr>
<tr>
<td>Do you use a filter to get rid of other bacterial contaminants?</td>
<td>50</td>
</tr>
<tr>
<td>What is the pore diameter?</td>
<td></td>
</tr>
<tr>
<td><strong>Atmosphere</strong></td>
<td></td>
</tr>
<tr>
<td>What is the atmosphere during incubation (gas mixture in percentages)?</td>
<td></td>
</tr>
<tr>
<td>How do you achieve this atmosphere? (e.g. commercial gas packs, gas replacement system)</td>
<td></td>
</tr>
<tr>
<td><strong>Identification</strong></td>
<td></td>
</tr>
<tr>
<td>What tests do you use to confirm <em>Campylobacter</em>?</td>
<td></td>
</tr>
<tr>
<td>Motility</td>
<td>85</td>
</tr>
<tr>
<td>Gram staining</td>
<td>92</td>
</tr>
<tr>
<td>Catalase</td>
<td>92</td>
</tr>
<tr>
<td>Oxidase</td>
<td>86</td>
</tr>
<tr>
<td>Growth temperature (please give specifications)</td>
<td>69</td>
</tr>
<tr>
<td>Hydrogen sulphide production</td>
<td>77</td>
</tr>
<tr>
<td>Sodium chloride tolerance</td>
<td>54</td>
</tr>
<tr>
<td>Sensitivity for antibiotics (please specify what antibiotics)</td>
<td>77</td>
</tr>
<tr>
<td>By what test(s) do you differentiate between both subspecies?</td>
<td></td>
</tr>
<tr>
<td>Glycine 1% (please give specifications)</td>
<td>62</td>
</tr>
<tr>
<td><strong>Additional tests</strong></td>
<td></td>
</tr>
<tr>
<td><strong>Immunofluorescence</strong></td>
<td></td>
</tr>
<tr>
<td>Do you use immunofluorescence for the detection of the bacterium?</td>
<td>27</td>
</tr>
<tr>
<td>Do you use immunofluorescence to confirm <em>C. fetus</em> once strains are isolated?</td>
<td>23</td>
</tr>
<tr>
<td>Do you prepare antibodies according to the protocol in the OIE Manual?</td>
<td>12</td>
</tr>
<tr>
<td>Alternatively, is the conjugate provided by a commercial company? (please specify what company)</td>
<td>15</td>
</tr>
<tr>
<td>Do you prepare your conjugates according to the protocol provided in the OIE Manual?</td>
<td>4</td>
</tr>
<tr>
<td><strong>Serological tests</strong></td>
<td></td>
</tr>
<tr>
<td>Do you use the vaginal mucus agglutination test?</td>
<td>35</td>
</tr>
<tr>
<td>Do you perform the test according to the protocol provided in the OIE Manual? If no, please specify (protocol or reference)</td>
<td>19</td>
</tr>
<tr>
<td>Do you use an enzyme-linked immunosorbent assay (ELISA)?</td>
<td>12</td>
</tr>
<tr>
<td>Do you prepare the antigen as described in the OIE Manual? If no, please specify (protocol or reference)</td>
<td>4</td>
</tr>
<tr>
<td>Do you perform the ELISA as described in the OIE Manual? If no, please specify (protocol or reference)</td>
<td>12</td>
</tr>
<tr>
<td>Do you use a serological test for individual animals? If yes, what test?</td>
<td>12</td>
</tr>
<tr>
<td>How do you make a selection of animals within a herd to determine the status of the herd?</td>
<td></td>
</tr>
</tbody>
</table>
Results

Nineteen percent of the responding laboratories reported that they performed no pre-treatment of the samples, 8% did not answer this question, and the remaining 73% of respondents reported that they at least performed a centrifugation step on the preputial washings. Other methods were reported less frequently – 38% use membrane pre-filtration, and 8% (n = 2) use liquefying vaginal mucus as a pre-treatment step.

Filtering of the samples

Reference

To reduce background growth of other bacteria in the samples, filtering of samples can be performed (12), but this may reduce the numbers of C. fetus (1). As the use of filters may decrease the sensitivity of the test, it is recommended to perform both direct plating and filtering prior to plating (5).

Results

About half of the laboratories perform filtering of samples prior to plating, and the pore-diameters used are 0.45 µm,
0.65 µm and 0.80 µm. It is not clear from the questionnaire whether these laboratories use filtering of the samples solely or whether they do this in combination with direct plating.

**Use of isolation media**

**Reference**

Several isolation media are described in the *Manual*.

**Results**

A wide variety of media are used for the isolation of *C. fetus*. Skirrow’s medium was mentioned most often (58%), yet over 20 additional media were also mentioned. The addition of antibiotics, blood, and growth supplements further increased the diversity of media used. Some countries reported the use of charcoal cefoperazone deoxycholate agar (CCDA).

**Culture atmosphere**

**Reference**

Gas replacement systems, candle jars, carbon dioxide (CO₂) incubators and commercial systems can be used.

**Results**

All described methods appeared to be used, ranging from candle jars, gas-pack pouches, and CO₂ incubators, to machines creating the desired atmosphere. The final gas-mixtures strongly differ between laboratories, yet the majority are within the recommended range that allows *C. fetus* to grow (2).

**Identification**

**Reference**

*Campylobacter fetus* can be identified by testing a combination of different biochemical properties.

**Results**

The majority of the laboratories perform tests described in the *Manual* (Table I). Gram-staining and testing catalase and oxidase activity are the most frequently used tests, followed by testing motility, hydrogen sulphide (H₂S) production and antibiotic sensitivity. The latter test is mainly focused on sensitivity to cephalotin and naladixic-acid, yet use of 15 different antibiotics was reported.

All laboratories grow strains at 37°C, but 69% of laboratories also grow bacteria at either 25°C and/or 42°C. In addition to the tests described above, most laboratories perform complementary tests, the most common of which are:

- API-campy
- selenite reduction
- nitrate reduction
- hippurate hydrolysis
- H₂S production (using a lead-acetate strip).

Other complementary tests that are used less frequently (they were reported just once each) are:

- indol test
- nitrite reduction
- glucose fermentation
- fatty acid composition
- morphology and colour of colonies
- enzyme-linked immunosorbent assay (ELISA) for serotype A or B.

Polymerase chain reaction (PCR) identification is carried out by a few laboratories in industrialised countries. Some reported that they were evaluating the use of this method for identification, using different target genes (Cif sapB2 [4, 7]).

**Subspecies differentiation**

**Reference**

The ability to grow in 1% glycine is regarded as the gold standard for the subspecies differentiation of *C. fetus*.

**Results**

Only 62% of the respondents use the glycine test for subspecies differentiation, and these countries reported different ways of executing the test. The composition of the basic medium used in this test differs between laboratories, but, more importantly, the exact concentration and preparation of the glycine also differs, some countries even reported the use of glycerol instead of glycine.

Alternatively to this biochemical subspecies differentiation test, a subspecies-specific PCR is used for discrimination by five (19%) of the responding laboratories.

**Immunofluorescence test**

**Reference**

The IFT has been described for both detection and identification of *C. fetus*. 
Results

Approximately one-third of the responding laboratories use IFT for either one of the purposes. About half of these laboratories obtain the antibodies commercially, whereas the other half produce their own. From the latter group less than 5% produces the conjugate by the method described by the Manual.

Serological tests/antibody detection

Reference

At present, tests for antibody detection against C. fetus include the vaginal mucus agglutination test and the ELISA.

Results

The first test is used by 35% of the respondents, and 19% of them use the procedure as described by the Manual. One of the respondents discouraged the use of this test for routine use owing to the high number of false positive and negative outcomes experienced, and because it appears to be useful on a herd basis only and not for single animal submissions.

The ELISA is performed as described in the Manual by 12% of the respondents.

Discussion

Animal health and trade

Animal health is an important issue for the agricultural production sector worldwide. Animal diseases can cause huge economic losses due to:

a) death of animals caused by infection
b) culling strategies when diseases are introduced into non-endemic areas
c) production loss
d) trade barriers when borders are closed for the import and export of animals, biological products (e.g. semen and embryos), and animal products for human consumption.

In the Netherlands and the UK, the economic impact of animal diseases was felt with the recent outbreaks of foot and mouth disease, classical swine fever and avian influenza (11, 13, 15), which emphasised the importance of disease control measures. As global trade in agricultural products is increasing (6, 14), there is a need for good disease control methods that are based on scientifically established data. The OIE and the Codex Alimentarius (Codex) are two of the three international standard-setting organisations specifically referred to in the Agreement on the Application of Sanitary and Phytosanitary Measures of the World Trade Organization (14); the Codex sets standards and guidelines for food safety and the OIE sets standards and guidelines for animals and their products (17, 18).


The OIE Code details the health measures to be used by the veterinary authorities of importing and exporting countries to avoid the transfer of agents that are pathogenic for animals or humans, while avoiding unjustified sanitary barriers. The Code is a reference document for use by veterinary authorities, import/export services, epidemiologists, and all those involved in the international trade in animal and animal products. The OIE Manual is the companion volume to the Code. The Manual describes internationally agreed laboratory methods for disease diagnosis and requirements for the production and control of biological products. It contains the most up-to-date tests for specific diseases and it can be used as a gold standard in veterinary diagnostic laboratories involved in veterinary health control programmes and clinical microbiology. The Manual is not a ‘fixed’ document but is updated regularly to include the ‘state-of-the-art’ diagnostic tests. The continuous update of the Manual depends on publications in peer-reviewed journals and on information about the tests used in Member Country laboratories. Insight into their practices ensures that the Manual reflects the work being carried out in laboratories. In this study, the authors evaluated the use of the techniques described in the Manual for the detection and identification of CfV – the causative agent of BGC.

Diversity in tests performed globally

The results of this inventory clearly showed the diversity of tests used for detection and identification of CfV. This is illustrated by the fact that not a single question was answered with 100% agreement among the respondents. It is possible that this diversity is even larger on the global scale, as in this study only a limited number of countries responded. Diversity in the methods used is expected because, in some cases, the Manual describes different methods for the same test. However, for several other methods, different tests were reported that are not described in the Manual. Diversity was noted for most parameters involved in the detection and identification of CfV, but was especially found in media, identification tests and subspecies differentiation. For example, differences in the composition of the media can influence the growth of
the strain on the media, and thus can have major consequences for the isolation of the agent. The use of CCDA was reported several times despite the fact that a substantial number of C. fetus strains are unable to grow on this media; the use of CCDA will thus result in under-reporting of the incidence. The diversity of methods used for collection, treatment, transport, enrichment, isolation, filtration, culture conditions, and identification tests may influence the sensitivity of the tests, possibly leading to trade disputes. However, it is unclear to what extent this diversity affects the outcome of the tests. Future work may focus on this by, for example, setting up ring-trials.

In the absence of a description of the performance of new techniques compared with the described techniques, it remains unclear as to what level the new techniques are an improvement on the old techniques. It is therefore strongly recommended that laboratories that introduce new techniques, evaluate the technique and report their findings in an internationally peer-reviewed journal. Following publication, these techniques could then be included in a next edition of the Manual.

**Monitoring the presence of Campylobacter fetus subsp. venerealis**

Diagnosis of clinical BGC can be difficult as bulls are usually subclinical carriers. Once the bacterium is introduced into a formerly uninfected herd, it may result in abortion and prolonged calving intervals because of early embryonic death. Diagnosis of the disease in herds with these nonspecific clinical signs can be hard and may take a long time. Because of this, the disease is sometimes referred to as ‘the quiet profit-taker’ (16). To control the disease, it is essential to monitor the causative agent of the disease as part of a veterinary health programme. Monitoring of the bacterium should be performed according to science-based protocols. Currently, there is a lack of elementary knowledge, such as the duration of carriership and the sensitivity of diagnostic tests, and thus the information published by the OIE on the occurrence of Cfv, compiled using Member Country reports, may not be complete.

**Subspecies differentiation**

An important aspect of BGC monitoring is the occurrence of the two subspecies, Cfv and Cfif. Although very similar, the two subspecies can be distinguished on clinical presentation. As only subspecies Cfv is regarded to be the causative agent of BGC, it is extremely important that the two subspecies be discriminated in a reliable way. However, the fact that subspecies differentiation is not widely applied indicates that, in some countries, no difference is made between the subspecies. This could result in needlessly introducing a trade barrier or even introducing the bacteria into a country with a Cfv free status. Both actions can have major economic and veterinary health consequences and therefore proper methods of subspecies differentiation are very important.

**Molecular methods**

From the evaluation it became clear that only a limited number of industrialised countries perform molecular methods for the typing or subspecies differentiation of C. fetus. It is possible that in time new molecular methods will be developed and currently available molecular methods will be improved. Improvements may include increasing the sensitivity of the tests and directly applying them to specimens to reduce analysis time. Although molecular methods have the potential to fully replace classical methods, it seems unlikely to happen within the near future as developing countries often lack the required resources to implement these techniques.

**Limitations of the inventory**

The limited number (16%) of returned questionnaires may give a distorted view of the methods used globally for the detection and identification of C. fetus. However, as the questionnaires were returned by countries with a wide geographical distribution, it is possible that, despite the small number, a good representation of the tests performed globally was obtained.

The limited response may have been partly caused by a language barrier, as the questionnaire was sent in English only. This language barrier may also have affected the interpretation of and response to the questions. Although most of the questions were formulated in a manner that makes misinterpretation unlikely, this cannot be ruled out. Some countries may not have returned the questionnaire because of limited resources and infrastructure within their Veterinary Services. Another possible reason for the limited response is that some countries do not have a Cfv screening programme because they are not involved in trade and do not report clinical BGC. Finally, as there was only one questionnaire per country, the data collected may not provide all the relevant details. Although it can be assumed that the respondent filled out the questions according to his/her best knowledge, other national or local laboratories may also be involved in screening programmes but may not have been involved in completing the questionnaire. Often laboratories involved in screening programmes are not the same laboratories that are involved in typing of isolates, and vice versa.
Conclusion

A diversity of methods used for the diagnosis of BGC has been reported, and most likely these differences result in variable sensitivity of the tests used. It is important to minimise the variation, and therefore it is recommended that laboratories perform tests according to standard procedures. When improvements to the tests are evaluated, laboratories are requested to publish their results in an international peer-reviewed journal so that these techniques could subsequently be included in the next edition of the Manual. Future improvements may, for example, focus on determining the effect of the diversity within tests used (e.g. setting up ring-trials), or on further standardisation of the methods (e.g. introduction of well-described control strains into the described protocols).

Acknowledgements

The authors thank Eric Gogstad (Centers for Disease Control and Prevention, Atlanta, USA) for supplying the digital global map.

M.A.P. van Bergen, S. Linnane, J.P.M. van Putten & J.A. Wagenaar

Détection et identification de Campylobacter fetus sous-espèce venerealis au niveau mondial

Mots-clés
Detección e identificación de *Campylobacter fetus* subesp. *venerealis* en el mundo

M.A.P. van Bergen, S. Linnane, J.P.M. van Putten & J.A. Wagenaar

**Resumen**
La campilobacteriosis genital bovina causada por *Campylobacter fetus* subesp. *venerealis* (Cfv) es una infección genital que amenaza al sector ganadero. La detección e identificación del microorganismo son dos elementos básicos de los programas de lucha contra esta enfermedad. Las normas comerciales deben basarse en métodos de detección e identificación de Cfv científicamente contrastados e internacionalmente aceptados, como los que se describen en el *Manual de pruebas de diagnóstico y vacunas para los animales terrestres* de la Organización Mundial de Sanidad Animal (OIE). Los autores realizaron un estudio para determinar los métodos utilizados en los Países Miembros de la OIE y tener así una visión general de las pruebas nuevas o mejoradas que se estaban aplicando. Para ello distribuyeron un cuestionario entre los 166 Países Miembros, de los que fueron devueltos 26. A escala mundial se observa una gran heterogeneidad en los métodos utilizados para detectar e identificar al agente causal de la enfermedad. Los autores llegan a la conclusión de que falta armonización, cosa que puede influir en la descripción de la situación sanitaria de un país y generar controversias respecto a las normas comerciales.

**Palabras clave**

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**References**


