SELECTIVE CULTURE MEDIUM TO ISOLATE *CLOSTRIDIUM SPIROFORME* FROM RABBIT GUT

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ABSTRACT

Enteritis due to *Clostridium spiroforme* is an emerging problem of intensive rabbit breading. To control the infection a laboratory support is needed both to diagnose the clostridiosis and to target the therapy. The aetiology can be suspected on the basis of the typical lesions and the bacterioscopical examination of the large intestine content. The isolation of *Clostridium spiroforme* is necessary to confirm the diagnosis and to evaluate the drug susceptibility. Isolation of *Clostridium spiroforme* is quite difficult because it requires a rich culture media and needs strictly anaerobic conditions; the microrganism has a low surviving time and produces small colonies not easy to identify. Moreover the isolation can fail because of overgrowing of other anaerobic or facultative anaerobic species (i.e. *Proteus* spp.). Thus the isolation procedure is too related to the subjective ability of the technician. To avoid the contamination problems a selective culture media (SM) to isolate *Clostridium spiroforme* was standardized. The commercial PAB medium (Perfringens Agar base,Oxoid) was supplemented with SFP (Shahidi-Ferguson-Perfringens Selective Supplement, Oxoid) and added with a 5% of sheep red blood cells, 500 µg/ml of tylosin (Tylosin tartrate, Sigma) and 12,5 µg/ml of rifampicin (Rifampicin powder, Sigma). The SM was tested with 105 samples of different clinical significance and compared with the control medium. Results evidenced the ability of the SM to isolate *Clostridium spiroforme* in 79% of samples. Best results were obtained with samples without chemical and physical pre-treatment, so that it was enhanced the sensitivity of the medium while reducing both time and tools consuming. Moreover the capacity of the medium to isolate pure colonies from samples of high clinical relevance (47.5% of isolation against 13.3% of control media) reduces the necessity of specialized personal and makes the procedure easier to standardize.

Key words: *Clostridium spiroforme*, rabbit, selective medium

INTRODUCTION

Despite improvements in technological tools, management and hygiene, digestive diseases still represent the main sanitary problem of rabbit breeding. Clostridial enteropathies are increasing as a consequence of the massive use of antibiotics, which by changing the caecal flora promote clostridiosis outbreaks (CARMAN and BORRIELLO, 1984).

In the past *Clostridium perfringens* was mostly involved in rabbit clostridiosis disease while recently *Clostridium spiroforme* aetiology is increasing (AGNOLETTI F., data not published). The diagnosis of enterotoxaemia due to *Clostridium spiroforme* is quite easy to perform; it can be suspected on the base of clinical and macroscopical findings (rapid onset of diarrhoea, oedema, suffusions and petechia of the cecum wall) which are quite indicative of the pathology (figure 1-a). The microorganism has a typical shape (figure 1-b), by which it can clearly be distinguished after Gram staining of the cecal content (BORRIELLO *et al.,* 1986). The scheme described by PEETERS *et. al. (*1995) may also be used to quantify *Clostridium spiroforme* in specific clinical cases in order to evaluate the etiological role.

To confirm the diagnosis and to evaluate the drug susceptibility of *Clostridium spiroforme* it is necessary to isolate the bacterium in pure cultures.

Clostridium spiroforme is considered a fastidious microorganism as it requires fertile and pre-reduced culture media, strictly anaerobic condition and has a low survival time. To reduce the non-sporing germs contamination of the sample a chemical or physical treatment (BORRIELLO and CARMAN, 1983; CARMAN and BORRIELLO, 1983; HOLMES *et al*. 1988; KORANSKY and ALLEN, 1978; YONUSHONIS *et al*., 1987) helps in the isolation of pure cultures but is quite time consuming and dose not avoid the overgrowing of sporing germs other than *Clostridium spiroforme*. As a consequence the procedure is still too related to a subjective ability of the technician. In order to standardise the isolation method of *Clostridium spiroforme a* selective culture medium (SM) has been set up and evaluated in our Laboratory.

MATERIAL AND METHODS

Selective Medium production

To isolate *Clostridium spiroforme the* PAB medium (Perfringens Agar Base, Oxoid) was supplemented with SFP (Shahidi-Ferguson-Perfringens Selective Supplement, Oxoid) and added with a 5% (v/v) of sheep red blood cells. Finally the medium was added with 500 µg/ml of tilosin (Tylosin tartrate, Sigma) and 12,5 µg/ml of rifampicin (Rifampicin powder, Sigma). Our data and the literature reports (CARMAN and WILKINS, 1991)

indicate these two antibiotics were ineffective against *Clostridium spiroforme* but were able to reduce the growth of contaminating microorganisms.

Samples

Samples were 2 ml of cecum content of rabbits with enteritis. For each sample it was quantified the *Clostridium spiroforme* amount by an evaluation of the bacterium cells number identified by means of the Gram staining and the round or spiral shape. The scheme previously described (PEETERS *et al*., 1995) was used. Briefly, 0 means *Clostridium spiroforme* presence in less than 4 microscopic fields; 1+: the presence of isolated bacterium cells in 4-10 microscopic fields; 2+: the presence of several bacterium cells in several microscopic field, some of which producing a spiral chain and 3+: the presence of grouped bacterium cells in all microscopic fields.
One hundred and five samples with the following quantification: 30 samples as 0; 20 samples as 1+; 33 samples as 2+; 22 samples as 3+ were used to evaluate the SM.

SM evaluation

Each sample was pre-treated with 2 ml of absolute ethyl alcohol (alcohol treated – AT sample) or with the same amount of Brain Heart Infusion Broth (BHI, Oxoid), the latter represented the control as not alcohol treated (NAT) sample. Both samples were incubated at room temperature for 1 hour. To evaluate the efficacy, 105 AT and NAT samples were inoculated onto SM. Results were compared with the same AT and NAT samples inoculated onto the control culture medium (CM) represented by Columbia Agar (Oxoid) added with 10% (v/v) of sheep red blood cells (BORRIELLO and CARMAN, 1983; PEETERS *et al*., 1996). A 100 µl volume of sample was used. The SM and CM were used without pre-reduction.

Inoculated medium were incubated in anaerobic atmosphere (80% N_2, 10% CO_2 and 10% H_2) at 37°C for 48 hours. Petri plates were then examined for *Clostridium spiroforme* colonies: small, round, convex, with clean side and not haemolytic. Subcultures were checked for purity by aim of the microscopic examination.

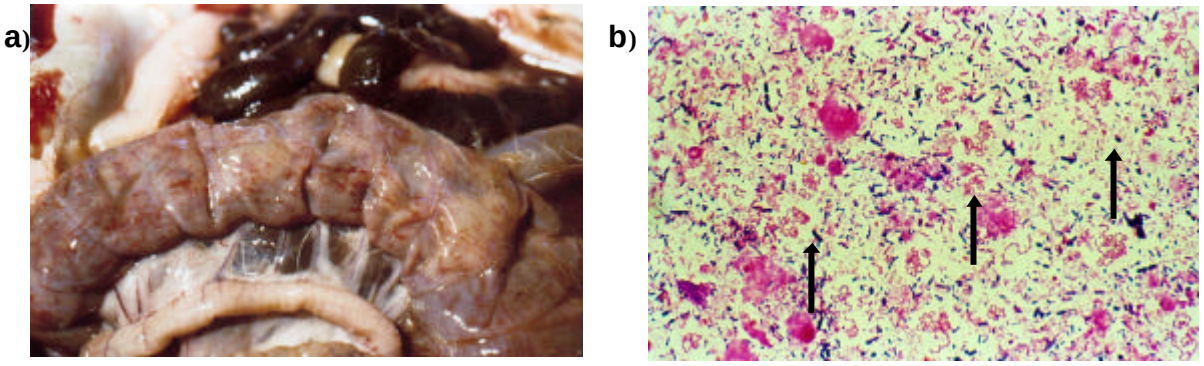

Figure 1. a) Rabbit gross intestine with lesions due to *Clostridium spiroforme* (oedema and haemorrhages of the intestine wall). b) Gram staining of cecum content: the typical round and spiral shape of *Clostridium spiroforme* is evidenced.

RESULTS AND DISCUSSION

Results of selective culture medium ability to support *Clostridium spiroforme* growth and inhibit other anaerobe and facultative anaerobe bacteria present in the cecum content of rabbits are expressed as percentage of isolation and summarised in the following tables. As expressed in Table 1 using the CM, *Clostridium spiroforme* was isolated in 41.8 % of NAT and 34.2 % of AT samples. The isolation procedure with SM was able to isolate the microorganism in 79% and 66.6% of respectively NAT and AT samples. The higher isolation rate of NAT samples is probably due to the reduction of live cell of *Clostridium spiroforme* in AT inoculums. Thus SM seems to be able to support the growth of the targeted bacterial strains quite twice as much as in the control media, it does -not make the chemical and physical treatment of the sample necessary and the procedure of isolation resulted more sensitive.

Table 1. Percentage of isolation (N. of isolation/N. of examined samples) of *Clostridium spiroforme* using the selective culture medium (SM) and the control culture medium (CM). Samples were alcohol pre-treated (AT) or not (NAT).

Medium	CM		SM	
Samples	NAT	AT	NAT	AT
N. samples	55[1]	105	105	105
% of C.spiroforme isolation	41.8	34.2	79	66.6
% failure of isolation[2]	3.6	2.8	0	0

[1] Results of 50 out of 105 tested samples were not available.
[2] *Proteus* spp. overgrowing.

Clostridium spiroforme is normally present and can be isolated from the rabbit large intestine and only in some circumstances can multiply and produce enterotoxaemia (PEETERS *et al*., 1986; SONGER, 1996). To be pathologically significant *Clostridium spiroforme* must be present in 2+ and 3+ amount. Results of 2+ and 3+ cells samples inoculated onto the SM evidence that it was possible to isolate *Clostridium spiroforme* in respectively 90.9% and 86.3% of NAT samples and 75.7% and 81.8% of AT samples. The isolation rate of *Clostridium spiroforme* onto CM was ranged between 45.5% of AT and 57% of NAT samples (Table 2).

Table 2. Evaluation of SM with samples (NAT and AT) of different clinical relevance N. of examined samples is indicated inside brackets. Results are expressed as percentage of *Clostridium spiroforme* isolation.

Evaluation of *C.spiroforme* cells in the cecum content	CM		SM	
	NAT	AT	NAT	AT
0	37.7 (14)	13.3 (30)	66.6 (30)	40 (30)
+	11.1 (9)	35 (20)	70 (20)	75 (20)
++	57.9 (19)	45.4 (33)	90.9 (33)	75.7 (33)
+++	46.1 (13)	45.4 (22)	86.3(22)	81.8 (22)

Normally one of the main problems encountered during *Clostridium spiroforme* isolation is the contamination with *Proteus spp.* Which covers the whole surface of the solid medium and prevent the isolation of other bacterial species. The isolation of *Clostridium spiroforme* could not be performed in 3,6% of NAT and 2,6% AT samples as a consequence of a *Proteus spp*. overgrowing. No *Proteus sp.* overgrowing was observed in plates of SM.

Finally with the SM it was possible to isolate *Clostridium spiroforme* in pure colonies in 47.5% of AT samples and 12.8% of NAT samples, compared with CM which was able to isolate pure cultures of *Clostridium spiroforme* with a lower rate (13.3%) and only from AT samples (table 3). The possibility to obtain pure colonies with the SM may be of importance when a drug susceptibility test is urgently required because the sub-cultivation of the microorganism is avoidable.

Table 3. Percentage of isolation of pure colonies from the samples with different *Clostridium spiroforme* cells amount. (N. of examined samples is indicated inside brackets).

Evaluation of *C.spiroforme* cells in the cecum content	CM		SM	
	NAT	AT	NAT	AT
0	0 (14)	0. (30)	0 (30)	42.8 (30)
+	0 (9)	0. (20)	33.3 (20)	33.3 (20)
++	0 (19)	22.2 (33)	12.5 (33)	53.3 (33)
+++	0 (13)	0. (22)	18.1 (22)	50 (22)
Total of pure isolation	0 (55)	13.3 (105)	12.8 (105)	47.5 (105)

CONCLUSIONS

Enteritis due to *Clostridium spiroforme* is going to be an emerging problem of intensive rabbit breading. The typical shape of the pathogen supports the diagnosis of this clostridiosis but the isolation of pure cultures is necessary although difficult to perform. The micro-organism is a fastidious one and requires a 10% concentration of sheep red blood cells in traditional medium and strictly anaerobic conditions; it has a low surviving time and produces colonies with no haemolysis halo and not easy to be recognised on the agar surface. Moreover the overgrowing of other anaerobic or facultative anaerobic species (i.e. *Proteus sp.)* makes the isolation a subjective ability of the technician. Aim of the present work was to formulate and evaluate a selective culture medium.

The isolation procedure by aim of the SM appears to be able to isolate *Clostridium spiroforme* in samples of clinical relevance in high percentages. The chemical and physical pre-treatment of samples can be avoided, unless a rapid drug evaluation test is required. The latter ability enhances the sensitivity of the medium while reducing both time and tools consuming. The selective culture medium permitted the isolation of the microorganism in pure cultures in a high percentage (90.9% - 86.3%) thus reducing the necessity of expert personal and making the procedure easier to standardise.

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