World Rabbit Science

World Rabbit Sci. 2009, 17: 185 - 193

INOCULATION AND BACTERIAL ANALYSES OF FRACTIONS OBTAINED FROM THE REFERENCE INOCULUM TEC4 WHICH EXPERIMENTALLY REPRODUCES EPIZOOTIC RABBIT ENTEROPATHY

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Abstract: The aetiology of epizootic rabbit enteropathy (ERE) is still unknown despite ten years of continuous research. A putative bacterial aetiology is the basis of current research. The fractionation of the reference inoculum (TEC4) is a major step towards finding the potential bacterial agent(s). In this study, TEC4 was fractionated by different techniques: centrifugation on discontinuous sucrose gradient, cell adherence and chloroform/ethanol treatment. The different fractions were inoculated into SPF rabbits and analyzed with classical bacteriological techniques. ERE was reproduced with two of the six fractions obtained. Four species never previously cultured from TEC were identified in the process but, to date, none of them seems to be the aetiology of ERE.

Key Words: Epizootic rabbit enteropathy, bacteria, fractionation, adherence.

INTRODUCTION

Epizootic rabbit enteropathy (ERE) has been spreading throughout Europe since 1997 (Duval, 1998; Marlier and Vindevogel, 1998). This digestive pathology induces several non specific clinical symptoms, including: decrease in food intake and water consumption, high mortality rate, and aqueous diarrhoea of limited extent. At necropsy, lesions are more typical. An overfull anterior digestive tract is consistently observed, including the stomach, with liquid and gas but without inflammation (Licois *et al*., 2005). Many treatments have been used, but to date only two antibiotics with market approval in most countries in Europe, zinc bacitracine and tiamulin, seem able to stop the clinical symptoms. Unfortunately, the symptoms often return after the end of treatment (Maertens *et al*., 2005). ERE costs producers approximately €10m yearly in Belgium and is a major cause of death in the European rabbit industry (Dewree *et al*., 2003).

The first hypothesis on the aetiology of ERE involved food poisoning and has now been fully ruled out (Le Gall *et al*., 1998; Lebas, 1998). Several studies have demonstrated that ERE is an infectious disease, since it can be reproduced by experimental inoculations (Licois, 1998; Licois and Coudert, 2001; Licois *et al*., 2005; Marlier *et al*., 2006). The French reference inoculum is called TEC (Licois and Coudert, 2001).

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Received *December 2008* - Accepted *May 2009*

Several generations of TEC have been produced: TEC, TEC1, TEC2, TEC3 and TEC4. The last one (TEC4) is actually the French reference inoculum (Licois and Coudert, 2005). A putative viral aetiology was believed to exist because of the quick spread of ERE. No specific virus has been found to date (Licois and Coudert, 1999; Marlier *et al*., 2003b; Licois *et al*., 2005). At least 2 factors of different sizes are involved in ERE pathogenesis. The first one has a size below 0.4 µm and the second over 0.4 µm. There is strong evidence that these factors are respectively a toxin and a bacterium (Marlier *et al*., 2003a; Coudert and Licois, 2004; Licois, 2007; Szalo *et al*., 2007).

Despite all the studies that have been carried out, the putative bacterial species causing ERE is still unknown. Either this species is not cultivable on traditional media, or its *in vitro* growth is inhibited by certain factors, including other bacterial species present in TEC. As Szalo *et al*. (2007) have suggested, the aspecific bacterial flora of TEC4 needs to be reduced before further analyses.

This study followed the basic centrifugation method developed by Szalo *et al*. (2007) followed by another fractionation based on adherence of bacteria to cells in a culture, as adherence to host tissues is a crucial step in the pathogenesis of many bacteria (Niemann *et al*., 2004). The bacterial composition and virulence of the new fractions produced were studied. They were then inoculated into rabbits and analysed with traditional bacteriology and bacterioscopy methods. The aim was to find a fraction still able to reproduce ERE but containing a limited amount of bacterial flora.

MATERIALS AND METHODS

Centrifugation on discontinuous sucrose gradient

TEC4 was first fractionated by the technique described by Szalo *et al*. (2007). Briefly, 1 mL of TEC4 was overlaid on 5 layers of 2 mL of each sucrose solution (10%, 20%, 30%, 40% and 50%) then centrifuged (1780 *g*, 4°C, 20 min). Seven fractions (supernatant, 10%, 20%, 30%, 40%, 50% and pellet) were collected and washed twice in 0.9% NaCl by centrifugation (20,000 *g*, 4°C, 3 min). Finally bacteria were resuspended in 0.5 mL NaCl 0.9%.

Adherence assay

Bacteria from the 50% centrifugation fraction were used for a second fractionation using rabbit kidney cells (RK13 cells). RK13 cells were grown in Dulbecco Modified Eagle's Medium (DMEM) supplemented with 100,000 units/L penicillin, 100 mg/L streptomycin sulphate, 5 mL/L MEM (100×) non-essential amino acids (Invitrogen), 2.5 mg/L natamycin and 5% foetal bovine serum. Twenty-four hours before RK13 cell line inoculation, the culture medium of a tissue culture flask (75 cm^2) at confluence was changed for growth medium without antibiotic. Twenty-four hours later, the culture medium was discarded and cells were inoculated with 1 mL of the 50% bacterial suspension. An RK13 cell-free culture flask was used as negative control. After 4 h at 37°C, culture and empty flasks were rinsed three times with sterile PBS to wash out non adherent bacteria. Cells and adherent bacteria were then recovered with a cell scraper and suspended in 1 mL of antibiotic free medium. These fractions were respectively named RK501 (RK13 cells) and PLA (RK13 cell-free flask) and stored at −20°C. The same fractionation plan was used in a second passage using RK501 adherent bacteria as inoculum. This fraction was named RK502 and stored at −20°C. The PBS used for the last wash before scrapping of RK502 fraction was kept, named RIN and stored at −20°C.

Chloroform/ethanol treatment

A solution of chloroform/ethanol (1/4 v/v) was used to reduce RK501 bacterial flora. One mL of RK501 was mixed with 2 mL of the $CHCl_3$/ethanol solution, kept one hour at room temperature under constant

stirring, washed by centrifugation (20,000g, 4°C, 3 min) and resuspended in 1 mL 0.9% NaCl. This fraction was named CERK and stored at −20°C.

All the fractionations were made under aerobic conditions for approximately 2 h for the centrifugation and the chloroform/ethanol treatment and 4 h for inoculations.

Inoculations

Three series of inoculations were carried out. Two hundred and twenty-six 4 w old SPF rabbits from the "Plate-Forme d'Infectiologie Expérimentale" (PFIE), INRA, Nouzilly, France (Licois *et al*., 2005) were used. This husbandry is clinically negative for ERE.

In the first series, fifty-four rabbits were randomly allocated to 3 batches. After one week of acclimatization, batch one was inoculated per os with 100 µL/rabbit of the 50% sucrose gradient fraction, batch two with 100 µL/rabbit of RK501 and batch three with 100 µL/rabbit of SPF rabbit's caecal contents (negative control). Each rabbit was weighed at D-7, D-4, D0, D3, D6, D8, D10 and D13. The rabbits were observed daily for external signs of diarrhoea. Days of mortality were recorded.

In the second series seventy-six rabbits were randomly allocated to 4 batches. Eighteen rabbits were inoculated per os with 100 µL/rabbit of SPF rabbit's caecal contents (negative control), 21 with 200 µL/rabbit of RIN, 18 with 100 µL/rabbit of RK502 and 19 with 100 µL/rabbit of PLA. Each rabbit was weighed at D-7, D-4, D0, D1, D2, D3, D6 and D8. Clinical monitoring was the same as above.

The third series was performed on ninety-six rabbits randomly allocated to 5 batches. Nineteen rabbits were inoculated per os with 100 µL/rabbit of SPF rabbit's caecal contents (negative control), 19 with 200 µL/rabbit of RIN, 19 with 100 µL/rabbit of CERK, 20 with 100 µL/rabbit of 50% and 19 with 100 µL/rabbit of RK501. Each rabbit was weighed at D-7, D-4, D0, D3, D6, D10 and D13. Mortality and presence of diarrhoea were recorded each day. Clinical monitoring was the same as in series 1 and 2.

Morbidity and mortality of each batch were respectively calculated as follows: number of rabbits in the batch presenting a drop in average daily weight gain for several days over total number of rabbits in the batch and number of dead rabbits with ERE lesion over total number of rabbits in the batch.

Statistical analysis

For analysing ADWG, a mixed model (PROC MIXED of SAS, 2000) was used, according to a repeated measures design that takes into account the variation between animals and covariation within them. The model included the treatment, the day, and their interaction as fixed effects. Least square means calculated by the model were compared by a Student's t-test.

Bacteriology

TEC4, 50% and RK501 were analysed by classical bacteriological methods. Ten microlitres of each fraction were inoculated onto blood agar plates containing 5% sheep blood and onto Mc Conkey agar plates and incubated at 37°C aerobically and anaerobically for 24 to 48 h. Bacterial colonies were identified using standard procedure (Barrow and Feltham, 1993). Strains were further identified by RAPID ID 32 A, RAPID 20 E and API 20 STREP (BioMerieux) according to the manufacturer's instructions. All colonies present on the plates were counted and CFU/mL was calculated.

Bacterioscopy

Ten microlitres of TEC4, 50% and RK501 were smeared onto microscopic slides, fixed and submitted to Gram staining (Szalo *et al*., 2007). Smears were observed at a 1000× magnification and 3 pictures were taken randomly for each fraction. The observed bacteria were counted and classified according to their

shape and colorimetric properties in spores, small size Gram-positive bacilli, small size Gram-negative bacilli, medium size Gram-positive bacilli, medium size Gram-negative bacilli, large size Gram-positive bacilli, large size Gram negative bacilli, Gram-positive cocci and Gram-negative cocci.

RESULTS

RK501 and 50% inoculation

A significant drop in the average daily weight gain (ADWG) was observed (Figure 1) as soon as D3 in rabbits inoculated with RK501 ($P<0.01$) or 50% fraction ($P<0.01$). Seventeen rabbits out of 18 in both batches had a drop in their daily weight gain for several continuous days. Global morbidity rate was 94% for the two batches. Global mortality rates were 28% and 11% respectively for the batch inoculated with RK501 and 50% with first mortalities observed at D4 for both batches. At necropsy, typical ERE lesions were observed: dilated digestive tract, stomach included, with no inflammation process. The ADWG of the control group was as expected and no clinical symptoms were observed during the observation period.

RK502, RIN and PLA inoculation

A slight drop in ADWG was observed at D2 in all batches (Figure 2) except the negative control: RK502 ($P<0.05$), RIN ($P<0.05$) and PLA ($P<0.05$). All batches have an ADWG lower than the control batch: RK502 ($P<0.01$), RIN ($P<0.05$) and PLA ($P<0.001$). Two rabbits died during the observation period: one from the RK502 batch with typical ERE lesions and one from PLA batch without any typical lesion. None of the fifty-eight inoculated rabbits showed a continuous drop of ADWG for several days. The ADWG of the control group was as expected and no clinical symptom was observed during the observation period.

RK501, 50% sucrose, CERK and RIN inoculation

The significant drop of ADWG can be seen at D3 (Figure 3) in groups inoculated with the 50% sucrose ($P<0.01$) or RK501 ($P<0.01$) fractions. No drop was observed in rabbits inoculated with CERK or RIN. Overall mortality and morbidity rates were respectively 0% and 0% in the RIN and the CERK batch, 16% and 37% in the RK501 batch and 10% and 40% in the 50% fraction batch. All dead animals showed typical ERE lesions. ADWG of the control group was as expected and no clinical symptoms were observed during the observation period.

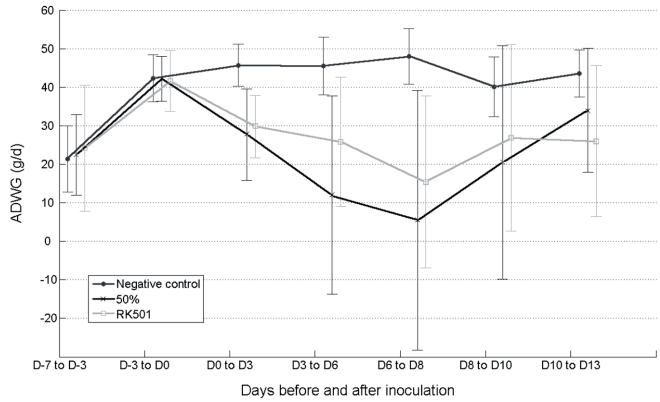

Figure 1: Comparison and evolution of average daily weight gain (ADWG) between the negative control batch and the two batches inoculated with the 50% sucrose and RK501 fractions (fractions as Table 2).

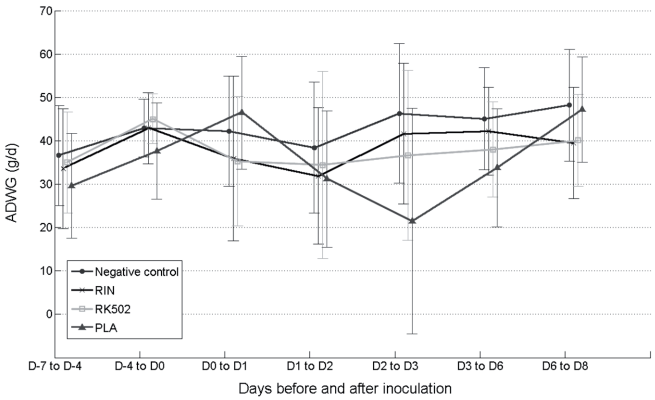

Figure 2: Comparison and evolution of average daily weight gain (ADWG) between the negative control batch and the three batches inoculated with the RIN, PLA and RK502 fractions (fractions as Table 2).

Bacteriology

All identified species and average CFU from virulent fractions are presented in Table 1. Average CFU of the virulent fractions showed a 10^2 decrease in CFU. Among the 9 species identified in TEC4, eight were also found in the 50% sucrose fraction. Of the 13 species detected in the 50% sucrose fraction, only 4 were also isolated from the RK501 fraction. Two other species were detected in the RK501 fraction but not in the 50% sucrose fraction or TEC4.

Bacterioscopy

Results of the bacterioscopic examinations are presented in Figure 4. A slight selection was observed through the first fractionation. Gram negative bacteria were absent in the RK501 fraction. Table 2 sums up the different characteristics of TEC4 and its fractions.

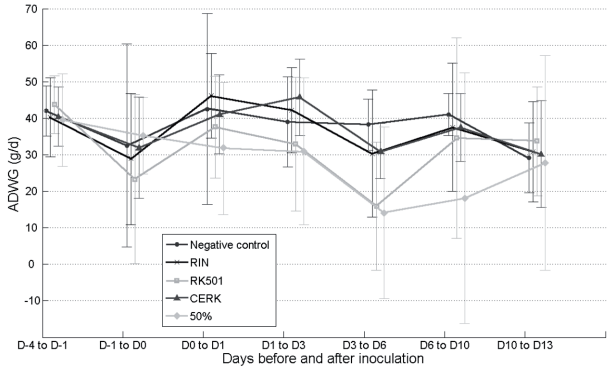

Figure 3: Comparison and evolution of average daily weight gain (ADWG) between the negative control batch and the four batches inoculated with the RIN, CERK, RK501 and 50% fractions (fractions as Table 2).

Table 1: Average CFU and bacterial species isolated from the TEC4, 50% sucrose and RK501 fractions plated in traditional culture media.

Fractions	TEC4	50% sucrose	RK501
CFU/mL	1,05.107	2,6.106	6,5.105
Species	Clostridium barati	Clostridium histolyticum	Sphingobacterium spiritivorum
		Clostridium sporogenes	Gemella morbillorum
		Clostridium acetobutylicum	
		Clostridium tyrobutyricum	
		Mannheimia haemolytica	
Species common to TEC4 and 50% sucrose fractions		Clostridium glycolicum	
		Clostridium sordelli	
		Bacillus sp	
		Brevundimonas vesicularis	
Species common to TEC4, 50% sucrose and RK501 fractions		Enterococcus faecium	
		Clostridium septicum	
		Clostridium fallax	
		Clostridium perfringens	

DISCUSSION

ERE is a major cause of loss in rabbit production but despite many research studies the aetiological agent is still unknown (Licois *et al*., 2005; Xylouri and Fragkiadakis, 2006; Dewrée *et al*., 2007). Parasites, fungi, toxics and viruses play no direct role in ERE according to previous studies (Lebas, 1998; Licois *et al*., 2005; Marlier *et al*., 2006). A potential bacterial aetiology is in agreement with the results of Szalo, *et al*. (2007) as well as the role of a putative toxin in the early phase of ERE (Marlier *et al*., 2003b; Licois, 2007).

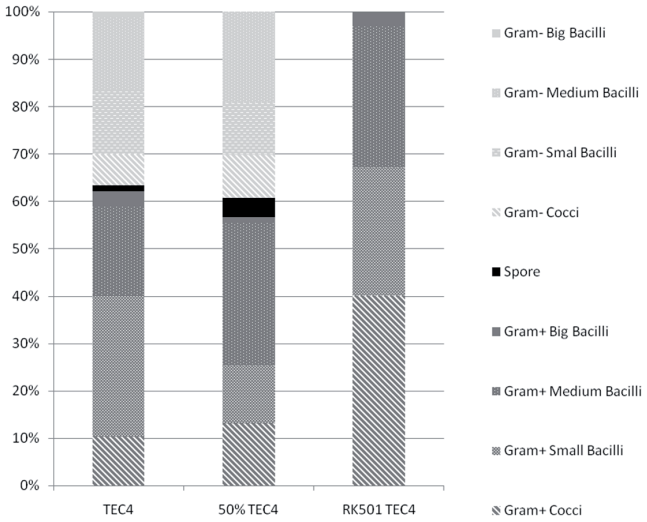

Figure 4 : Bacteria's repartition following stain and shape during the fractionation.

Table 2: Summary of the morbidity, mortality and bacterial flora obtained with the different treatments of the TEC4 inoculum.

Fraction	Making methods	Mobidity	Mortality	Flora diversity
50% sucrose	Centrifugation on discontinuous sucrose gradient	37 - 94%	16 - 28%	+++
RK501	First inoculation of the 50% fraction on RK13 cells	40% - 94%	10 - 11%	++
RK502	Second inoculation of the 50% fraction on RK13 cells	5,5%	5,5%	+
PLA	Inoculation of the 50% fraction on an empty flask	0%	0%	+
RIN	PBS used for the last wash before scrapping of RK502	0%	0%	+
CERK	Chloroform /ethanol treatment of the RK501 fraction	0%	0%	-

Fractionation of TEC seems necessary to identify the putative aetiological agent. As centrifugation of TEC3 on discontinuous sucrose gradient previously gave good results (Szalo *et al*., 2007), the same technique was used on TEC4. The 50% sucrose fraction was used in the second fractionation because it contains few bacterial cells and the aetiological agent is still at a high concentration (Szalo *et al*., 2007). Adherence to host cells being the first step in many bacterial pathologies (Niemann *et al*., 2004), it was hypothesized that the aetiological agent is most probably able to adhere to rabbit intestinal epithelial cells. As immortal rabbit enterocytes are not available, rabbit kidney cells (RK13) were chosen to make a fractionation of 50% by cell adherence.

Inoculations were made without any trouble except for a slight drop in ADWG on the third inoculation between D-1 and DO in all batches. This may be due to a high temperature gap between night and day, normal at this time of the year. Successful ERE reproduction was only possible with two of the six fractions. The concentration of the putative responsible bacteria in the 50% sucrose and RK501 fractions were still high enough to reproduce a typical ERE. In the other four fractions (RK502, PLA, RIN and CERK) the agent is probably still present, since a slight drop of ADWG was noticed but the concentration is not high enough to allow reproduction of the typical clinical signs and/or lesions. Similar ADWG evolution is seen with inoculation of 10^{-4} dilution of TEC4 (Licois and Coudert, 2005). ADWG is the most important sign of ERE experimental reproduction in TEC inoculation (Licois *et al*., 1998; Licois *et al*., 2005). However, gross lesions are the second confirmation of the reproduction. All dead animals of the 50% sucrose and RK501 fractions inoculation batches showed typical ERE symptoms: anterior digestive tract dilation with no inflammation. Other negative controls created from caecal content of healthy rabbits treated in the same way as the fractions could have been used to rule out a potential dysbacteriosis effect. However, the ADWG drop and, above all, the typical gross ERE lesions cannot be the consequence of a simple dysbacteriosis only.

The ERE aetiological agent is probably not cultivable on a conventional medium or inhibited by other microorganisms (Szalo *et al*., 2007). The aim of the fractionations is to reduce the bacterial flora. Conventional bacterial analyses were essentially made to evaluate fractionation efficacy. The bacterial flora of the three virulent fractions (TEC4, 50% sucrose and RK501 fractions) are very different both quantitatively and qualitatively. The total bacterial concentration (CFU/mL) and the number of bacterial species are successfully reduced. Surprisingly fewer species are identified in TEC4 compared to those of the 50% fraction. However, this can be explained by the high concentration of *Clostridium glycolycum*, which inhibits other species' growth. The number of cultivable bacterial species is lowest in the RK501 fraction. Only 4 of the 14 species found in TEC4 and 50% sucrose fractions are still present. Conversely, the limited bacterial flora of the RK501 fraction allows the detection of two other species undetected in the other fractions.

The bacterial flora of TEC has been determined in several studies (Licois *et al*., 2003; Licois *et al*., 2005; Marlier *et al*., 2006; Szalo *et al*., 2007). Four species that had not been detected in TEC to date were grown in common bacteriological culture from TEC4 and its new fractions: *Mannheimia haemolytica, Brevundimonas vesicularis, Sphingobacterium spiritivorum* and *Gemella morbillorum*. The first is a primary pathogen, causing pneumonia, mammitis and septicaemia in ruminants (Euzéby, 1998). Nevertheless, the identification percentage of the commercial gallery was poor (75.4 %) and this bacterium could also be any non pathogen Pasteurella like species. The other three new species are environmental bacteria and can be responsible for nosocomial infections (La Scola and Raoult, 1998; Hiar *et al*., 2002; Yang *et al*., 2006). Still, it seems unlikely that these species are the ERE aetiology, until proved otherwise.

In the bacterioscopic analyses, an increase in the spore concentration is observed confirming previous results (Szalo *et al*., 2007), even if the total amount of spores in TEC4 seems lower than in TEC3. On the other hand, a selection of bacilli was not observed in the 50% fraction. The lack of Gram negative bacteria from RK501 is surprising. The remains of the staining of the RK13 cells could mask the Gram negative bacteria, coloured in the same tone, although even if the Gram negative bacteria are not completely removed by the fractionation they would be present in very small quantities.

CONCLUSIONS

Although the aetiological agent was not identified, new information was obtained by these fractionations: the 50% sucrose fraction reproduced the pathology, confirming a potential bacterial ERE aetiology, probably a Gram positive bacterial species. New species were also cultured from the reference TEC4 inoculum. These new virulent fractions should be analysed by molecular biology techniques to avoid the limiting culture step.

Acknowledgments: This work was supported by a Grant from the «Service Publique Fédéral Santé Publique, sécurité de la chaîne alimentaire et environnement: Division Recherche contractuelle», contract RT 06/7 MINRABBIT. N. Huybens is a PhD fellow of the "Fonds pour la formation à la Recherche dans l'Industrie et dans l'Agriculture" (F.R.I.A.).

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