ORIGINAL ARTICLE

Multicausal etiology of the enteric syndrome in rabbits from Mexico

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Abstract Enteropathies in rabbits are difficult to diagnose; their etiology involves pathogens that act synergistically, causing damage to the intestine. The aim of the present study was isolate enteric pathogens from rabbits in Mexico. Using parasitological, bacteriological and molecular analyses, we screened 58 samples of the intestinal content of rabbits having a clinical history of enteric disease from the southeastern part of the State of Mexico. Out of the 58 samples analyzed, a total of 86 identifications were made, *Eimeria* spp. were found in 77.5%, followed by *Aeromonas* spp. in 15.5% and *Escherichia coli* in 8.6%, which were identified as enteropathogenic *E. coli* (EPEC), and the presence of the following agents was also confirmed: *Salmonella* spp., *Klebsiella* spp., *Streptococcus* spp., *Staphylococcus aureus*, *Enterococcus* spp., *Mannheimia* spp. and *Rotavirus*. The concurrent presence of *Eimeria* spp. with *Aeromonas* was frequent (15.5%); there was statistical significance for the presence of an association between the clinical profiles and *Eimeria* spp. ($p = 0.000$), *Mannheimia* spp. ($p = 0.001$), *Salmonella* spp., *Klebsiella* spp., *Streptococcus* spp. and *Enterococcus* spp. ($p = 0.006$).
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Multicausalidad del síndrome entérico en conejos de México

Resumen Las enteropatías en conejos son difíciles de diagnosticar, debido a que en su etiología participan patógenos que actúan en sinergia y causan daño al intestino. El objetivo de este estudio fue el aislamiento de patógenos de cuadros entéricos en conejos de México. Mediante métodos parasitológicos, bacteriológicos y moleculares, se analizaron 58 muestras

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de contenido intestinal de conejos con el antecedente de cuadro clínico entérico procedentes de granjas cunícolas de la zona suroriente del Estado de México. A partir de las 58 muestras se realizaron un total de 86 identificaciones, los patógenos más frecuentes fueron *Eimeria* spp. (77,6%), seguidas de *Aeromonas* spp. (15,5%) y de *Escherichia coli* (8,6%), identificadas como *E. coli* enterpatogénicas (EPEC). También se determinó la presencia de otros agentes: *Salmonella* spp., *Klebsiella* spp., *Streptococcus* spp., *Staphylococcus aureus, Enterococcus* spp., *Mannheimia* spp. y *Rotavirus*. La presencia concurrente de *Eimeria* spp. y *Aeromonas* spp. (15,5%) fue la observación más frecuente. Se encontró asociación estadísticamente significativa entre la presentación del cuadro clínico en conejos y la presencia de *Eimeria* spp. ($p = 0,000$), *Mannheimia* spp. ($p = 0,001$), *Salmonella* spp., *Klebsiella* spp., *Streptococcus* spp. y *Enterococcus* spp. ($p = 0,006$).

Introduction

The enteritis complex was introduced by Whitney in 1976 to designate a group of diseases that include mucoid enteritis, typhlitis, typhlitis-diarrhea and hemorrhagic enteritis. Commonly observed symptoms include teeth grinding, anorexia, polydipsia, weakness, abdominal distension, profuse diarrhea, dehydration, hypothermia and death[18]. Enteropathies are recognized as a problem for domestic rabbits, being their etiology and pathogenesis poorly understood[4]. In commercial rabbit production, enteropathies are hard to diagnose; they can have a multifactorial etiology (hence the term ''multifactorial enteritis'') and are also known as ''enteric syndrome''[14]. This syndrome is a pathological complex characterized by various stressing and pathogenic factors acting synergistically, possessing varying degrees of virulence which can enhance their pathogenicity and cause damage to the intestinal tissue. Numerous studies have reported the isolation of *Clostridium perfringens, Clostridium piliformis, Clostridium spiriforme, Escherichia coli*[21,35], *Salmonella* spp., *Klebsiella* spp., *Pseudomonas* spp., Streptococcus *aureus, Streptococcus* spp., *Campylobacter* and *Eimeria* spp.,[14,19,23,24]. Viruses seem to have an important but non-crucial role; overall, they are mild pathogens that do not induce severe clinical profiles[14]. Rotavirus[16], Coronavirus[4], and Parvovirus[17] have been reported and it has been suggested that they could exert direct pathogenic activity by initiating the development of bacterial infections and/or of other viral pathogens by inducing minimal alterations in the intestinal epithelium[17]. Enteric diseases are important in rabbit production and cause severe economic losses, due to high mortality indexes, decrease in growth and reduction in the feed conversion ratio[14]. Studies in Cuba, Japan and Argentina have reported digestive pathologies as the main cause of mortality with percentages of 57.9%, 48.9% and 43% respectively[24]. In Mexico there are few reports concerning the agents involved in these pathologies[39]. Because of this reason, the aim of this study was to isolate pathogens from enteric disease in rabbits from Mexico.

Material and methods

Fifty-eight rabbits with an enteric clinical profile were obtained from several rabbit meat production from the southeastern part of the State of Mexico, Mexico. The animals exhibited an enteric clinical profile that included depression, anorexia, dehydration, abdominal distension, liquid-to-mucoid diarrhea and death within 24 h. Isolates were recovered from the intestinal contents through the necropsy service of the Amphitheater of Veterinary Medicine and Zootechnics of the Centro Universitario Amecameca (CU Amecameca), of the Universidad Autónoma del Estado de México (UAEMex). This study was authorized by the Bioethics Committee of the CU Amecameca (CBE/06/2013).

Eimeria spp. were identified by microscopy and the parasitological flotation technique[29]. DNA was extracted from the stool samples using the ZR Fecal DNA MiniPrep™ (ZYMO Research, USA), according to the manufacturer's instructions. DNA samples were assessed quantitatively and qualitatively by measurements of absorbance in a Nano Drop 2000c (Thermo Scientific, USA). A PCR was also performed, using primers for the amplification of the ribosomal cistron, ITS1 region (400–500 bp long) of Eimeria tenella, as reported by Oliveira et al.[19] (accession number AFO26388). The following reagents were added to 1 mg of DNA to make 25 μl PCR solution: 2.5 mM MgCl$_2$, 100 mM each dATP, dCTP, dGTP, dTTP, 1.5 U GoTaq Flexi DNA Polymerase (PROMEGA, USA), 5 μl 5× Green GoTaq Flexi Buffer, and 0.8 mM of each primer. The amplification conditions included 35 cycles of denaturation at 96 °C for 45 s, annealing at 54 °C for 45 s and extension at 72 °C for 60 s. A sequenced sample was included as positive control and DNA obtained from rabbit blood was used as negative control.

The bacteriological analyses involved primary isolation in blood agar; identification was performed by colonial morphology and Gram staining. Gram negative bacteria were cultured in the following selective media: MacConkey, Salmonella-Shigella and brilliant green agar at 37 °C incubation temperature and aerobiosis for 24–48 h[2]. To identify *Salmonella*, the samples were previously inoculated in peptone water and tetrathionate broth. A colony of each

growth produced was selected as a representative isolate and subcultured for further biochemical identification. Biochemical tests of oxidase, catalase, triple sugar iron, ornithine decarboxylase, sulfide indol motility and urease were performed[5,10,14]. Gram positive bacteria were analyzed for evidence of catalase, coagulase, hemolysis, and cultured in mannitol salt agar[40].

To determine the *E. coli* pathotypes, we used a pathogen-specific multiplex PCR to detect enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC) and Shiga-toxin-producing or enterohemorrhagic *E. coli* (EHEC o STEC o VTEC), using specific primers, by the amplification of 150–650 bp fragments for diverse virulence traits as reported by López-Saucedo et al.[15] Representative positive PCR products were purified and sequenced using an ABI PRISM 3500 genetic analyzer (Life Technologies, Carlsbad, CA).

Rotavirus detection was accomplished by the amplification of a 380 bp fragment of capsid protein VP6, employing primers as reported by Iturriza-Gomora et al.[12] RNA extraction was performed using GeneJET Viral DNA and the RNA Purification Kit (Thermo Scientific, USA). The two-step RT-PCR technique was implemented; complementary DNA (cDNA) was obtained by means of the SuperScript III Reverse Transcriptase (RT) (Thermo Scientific, USA). PCR conditions were as follows: Initial denaturation at 96 °C for 10 min, follow by 45 cycles of denaturation at 96 °C for 45 s, annealing at 61 °C for 45 s and an extension at 72 °C for 30 s, the final extension at 72 °C for 5 min. As a positive control, we used the Rota teq pentavalent vaccine (MSD SNC, Lyon, France).

In order to test the association between the presence of the identified agents and the clinical profiles, we used the Fisher's exact test, with a significance threshold of ($p < 0.05$).

Results

Out of 58 intestine content samples, 35/58 (60.4%) were positive for at least one identification and 23 (39.6%) were in concurrence, including: 3/58 (5.2%) with two identifications and 14/58 (24.0%) with three identifications. Rotavirus was identified for the first time in rabbits from Mexico in 6/58 samples (10.3%), of which 5/6 samples (83.3%) showed the identification of concomitant infection by Rotavirus and other pathogens.

We identified six highly pathogenic *Eimeria* species in 45/58 rabbits (77.5%); mildly pathogenic *E. intestinalis* in 2/58 rabbits (4.4%) and *E. flavescens* in 2/58 (4.4%); lowly pathogenic *E. magna* in 35/58 (77.7%), *E. media* in 8/58 (17.7%); *E. perforans* in 12/58 (26.6%) and *E. vejdovsky* in 4/58 (8.8%), followed by *Aeromona* spp. (15.5%) and *E. coli* in 5/58 (8.6%), corresponding to enteropathogenic *E. coli* (EPEC) while the other strains were non-diarrheagenic by PCR, using primers for *bfp*A detection[15].

There were 86 identifications in the 58 samples. Figure 1 shows the frequency of detection per agent. The concurring presence of pathogenic agents identified in the intestine content samples is shown in Table 1. Results of the Fisher's exact test showed a statistically significant association of the clinical profiles and the presence of *Eimeria* spp. ($p = 0.000$), *E. coli* EPEC ($p = 0.09$), *Mannheimia* spp.
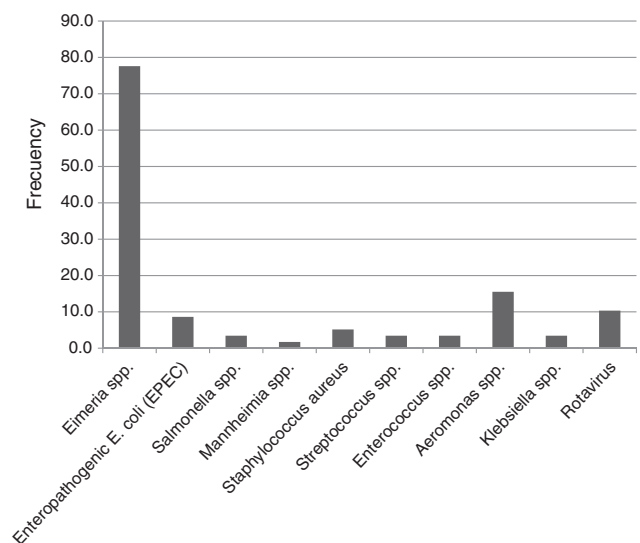
Figure 1 Agents isolated from enteric profiles in rabbits from Mexico.

($p = 0.001$), *Salmonella* spp., *Klebsiella* spp., *S. aureus* and *Enterococcus* spp. ($p = 0.006$) (Table 2). In relation to the clinical profile observed, diarrhea (with varying characteristics) was the most frequent sign, followed by abdominal distension.

Discussion

In contrast with the results obtained by Szalo et al.[34], who suggested that the enteropathy in rabbits is a bacterial disease and does not have a viral or parasitic etiology and with Martella et al.[16], where rotavirus was the most frequent agent isolated from enteric profiles in rabbits, in the present study, *Eimeria* spp. was the most frequently recovered agent, both on its own and in association with other pathogens. Studies in other countries have reported the presence of eleven *Eimeria* species that affect rabbits in up to 70% of enteropathies, and, according to their level of pathogenicity, can cause reduced growth rate and feed conversion, and increased mortality[19,21]. In this work we only identified by PCR six of the eleven *Eimeria* species in the samples, being *E. magna* and *E. perforans* the most frequently found, considered as mildly and lowly pathogenic respectively; however, although all the rabbits presented enteric disease, the association with other pathogens had not been reported. Interestingly in the present study, we found an association of *Eimeria* spp. with gram negative bacteria and less frequently with Gram positive bacteria. These results provide epidemiological evidence of synergism among *Eimeria* spp. and *E. coli* and other enteric pathogens. It seems that by being simultaneously present in the enteric profiles, these pathogens can increase their pathogenic potential[3].

The replication of *Eimeria* spp. within the epithelial cells of the intestine, alters the natural resistance barrier[30], which is intensified due to various factors such as the production of inflammatory cytokines, mainly interleukin 6 (IL-6) and to a lesser degree interleukin 8 (IL-8) released as a result of massive cell lysis. These mechanisms are used by

Table 1 Identification of concurring agents in rabbit intestine samples from Mexico.

Concurrent identification	Frequency	Percentage
Enteropathogenic *Escherichia coli* (EPEC)	5	8.7
Eimeria magna	25	43.1
Enterococcus spp.	2	3.5
Streptococcus spp.	1	1.7
Rotavirus	1	1.7
Mannheimia spp.	1	1.7
Eimeria magna + *E. perforans* + *Aeromona* spp.	9	15.5
Eimeria media + *Sthapylococcus aureus*	1	1.7
Eimeria media + *E. intestinalis* + *E. perforans* + *Staphylococcus aureus*	2	3.5
Eimeria magna + *E. perforans* + *E. vejdovsky* + *Streptococcus* spp.	1	1.7
Eimeria vejdovsky + *Klebsiella* spp.	1	1.7
Eimeria vejdovsky + *Salmonella* spp.	1	1.7
Eimeria media + *E. flavescens* + *Rotavirus*	2	3.4
Eimeria media + *E. vejdovsky* + *Rotavirus*	1	1.7
Eimeria media + *Klebsiella* spp. + *Rotavirus*	1	1.7
Eimeria media + *Salmonella* spp. + *Rotavirus*	1	17.0
Without isolation	3	5.2
	58	100.0

gram negative bacteria such as *E. coli* and *Salmonella* spp. to increase intestinal wall disorganization, consequently invading the tissue[6,22], facilitating bacterial adherence and penetration[27]. Further, some *Eimeria* species enhance host mucogenesis since the mucus layer of the gastrointestinal tract is an excellent source of nutrients for some bacteria[20]. Blankets and globular particles of mucus associated with numerous bacteria on villi and extensive inflammatory lesions have been observed in rabbits with an enteric profile and bacteria were observed attached to the site of serious cell damage[6]. They can take advantage of this scenario for synergies and exercise their mechanisms of pathogenicity or may be considered as secondary invaders.

Aeromonas spp. are members of the microbiota of warm-blooded animals and have been reported to cause septicemia and gastroenteritis (with self-limiting acute diarrhea)[1]. Their mechanism of pathogenicity is through type III secretion systems, with production of cytotoxic, hemolytic, enterotoxic and lethal enterotoxin, which also alters the cytoskeletal signaling cascades and promotes bacterial growth[25,28]. The pathogenic role of this bacterium in enteric disease has been demonstrated and studied in the present study, *Aeromonas* spp. occurred with a frequency of 10.47%. The isolation of *Aeromonas* spp., on their own or in association with other pathogens from the intestinal contents suggests their involvement in the enteric profiles.

In our study, enteropathogenic *E. coli* (EPEC) was found in five animals (8.6%), which is very similar to Swennes et al.'s findings[32,33], who described 10.5 and 4.3% EPEC in laboratory rabbits. Lavazza et al.[14] found that *E. coli* was the only pathogen in about 40.0% of cases of domestic rabbits. Dewrée et al.[6] found that the most commonly isolated digestive tract bacteria pathogens were *E. coli*, but they did not find EPEC. This bacterium is a member of the intestinal microbiota and possibly several factors generate a state of immunosuppression, which allows significant replication of some opportunistic agents or performing synergy with other competing agents[10,14,23,36].

Table 2 Comparison of rabbits positive and negative to enteropathogens from intestine samples from Mexico.

Pathogen	Positive	Negative	*p*
Eimeria spp.	45	13	0.000[a]
Enteropathogenic *E. coli* (EPEC)	5	53	0.09
Salmonella spp.	2	56	0.006[a]
Mannheimia spp.	1	57	0.001[a]
Staphylococcus aureus	3	55	0.084
Streptococcus spp.	2	56	0.006[a]
Enterococcus spp.	2	56	0.006[a]
Aeromonas spp.	9	49	0.14
Klebsiella spp.	2	56	0.006[a]
Rotavirus	6	52	0.11

Fisher's exact test, *p* < 0.05.
[a] Samples with statistical significance.

We found that 60.4% of intestinal content samples were positive for one identification, 5.2% for two and 24% for three. Other studies in human detected enteropathogens in 66.4% of patients with diarrhea, a single enteric pathogen in 50.9% and multiple pathogens in 15.5%; concomitant infection by more than one enteric pathogen occurred in 18.6% of the infants and 29% contained multiple pathogens[8].

The enteric syndrome has a large impact on rabbit production. Several authors suggest that rotavirus could be the main cause of enteric disease in rabbits and also be implicated as the etiological agent of severe enteric outbreaks[16,35]. Lavazza et al.[14] identified rotavirus in rabbits by electron microscopy (16.0%), detecting 3.3% to 36.6% in humans[8,27,37], our findings show the presence of rotavirus in fewer cases (10.3%). The concomitant identification of rotavirus and other pathogens was detected in 5/6 (83.3%) samples. *In vitro* models of pathogenesis indicate that

synergism between rotavirus and invasive bacteria involve specific biologic pathways: the attachment of, or the invasion through an up-regulation of specific receptors[31]. Bhavnani et al.[3] found epidemiologic evidence of synergism between rotavirus and other enteric pathogens for producing enteric clinical profiles, either by enhancing attachment and bacterial invasion of intestinal epithelial cells or by producing inflammation promoting the development of bacteria by the release of fluid, mucin, cellular detritus and the secretion of antimicrobials, which could alter the composition of the gut microbiota, allowing pathogens to occupy their commensal niche. Lavazza et al.[14], conclude that there was no association between viral positivity or negativity and the presence of bacteria. Our results agree with those of Dewrée et al.[6] and Rodriguez De Lara et al.[23], about rotaviruses as the main cause of the epizootic outbreaks of diarrheas is questionable, particularly if considering that rotavirus are considered endemic and mildly pathogenic in commercial rabbitries. However, this is the first molecular report confirming the presence of rotavirus in the enteric profiles of rabbits from Mexico.

In the bacteriological results in rabbits, Lavazza et al.[14] included *E. coli*, *S. aureus*, *Klebsiella* spp., *Yersinia* spp., Pasteurella *multocida, C. perfringens* and *C. spiriforme*. This study found *E. coli, Aeromonas* spp., *Salmonella* spp., *Enterococcus* spp., *Streptococcus* spp., *S. aureus*, *Mannheimia* spp., and *Klebsiella* spp. In humans, the results of several research works were similar: in Jordan, enteropathogenic *E. coli* (12.8%), enteroaggregative *E. coli* (10.2%), enterotoxigenic *E. coli* (5.7%), *Shigella* spp. (4.9%), *Entamoeba histolytica* (4.9%), *Salmonella* spp. (4.5%), *Campylobacter jejuni/coli* (1.5%), *Cryptosporidium* spp. (1.5%), enteroinvasive *E. coli* (1.5%), *Giardia lamblia* (0.8%) and *Yersinia enterocolitica* (0.4%)[39]; in Colombia, enteropathogenic *E. coli* (6.0%), enterohemorragic *E. coli* (2.8%), *Aeromonas hydrophila* (2.0%) and other pathogens (52.6%)[36] were identified; in the Netherlands, enteropathogenic *E. coli* were detected in 19.9%, and *Salmonella enterica* in 0.3%[8]; in Papua New Guinea, *Shigella* spp. (26.6%), enteropathogenic *E. coli* (8.5%), *Salmonella* spp. were found below the limit of detection[27].

Staphylococcus spp. make up the microbiota of the upper respiratory tract and lower urogenital tract, are transitory in the digestive tract and predominant in the small intestine and cecum; it has been observed that *S. aureus* produces yellow diarrhea in nursing rabbits[12]. Lavazza et al.[14] found *Staphylococcus* both in isolation and associated with *E. coli* and *P. multocida*; with and without viral presence. Furthermore, they found *Streptococcus* as single isolates from rabbits with enteritis. Our results show the presence of *S. aureus* in association with *Eimeria* spp. in 5.2%. *Streptococcus* spp., were found both on their own (1.7%) and in a concurrent manner with protozoa parasite *Eimeria* spp. (1.7%). Isolation frequency was not as high as in the case of other pathogen, however, their presence indicates involvement in promoting enteric profiles in rabbits.

Enterococcus spp. are ubiquitous and are consistently found in animal gastrointestinal tracts. In pigs, the co-occurrence of *Enterococcus* genus and *E. coli* has been previously reported and this interaction has been found to contribute to the development of new neonatal porcine

diarrhea[11]. Specifically, *Enterococcus hirae* has been associated with enteropathy in cats and with diarrhea in rats and nursing kittens[7,9,13]. It has also been isolated from an outbreak of diarrhea in nursing rabbits with 85% morbidity and 50% mortality; rabbits died within a 3 day period[38]. In the present study, we found *Enterococcus* spp., without any associated pathogenic agent in two samples of intestinal content (3.5%) related to diarrhea episodes, which suggests they could be etiologic agents.

Dewrée et al.[6] studied the epizootic rabbit enteropathy and found several bacteria adhering to the epithelial surface and inside the enterocytes in a few animals; none of the bacteria isolated from the intestinal mixed contents and cultivated on usual media were known as rabbit pathogens. There are several specific cases suggesting that pathogens can have significant impact upon each other, sometimes directly but at other times through the changes they cause to particular body systems and processes, including layers and components of the immune system[26,30]. The presence of several pathogens in more than half of the intestinal content samples of the rabbits with enteric clinical profiles required to determine if these pathogens found concomitantly can act synergistically, and interestingly, if these clinical enteric profiles are different when they are produced by a single pathogen and if the presence of two or more pathogens, increasing pathogenicity.

Ethical disclosures

Protection of human and animal subjects. The authors declare that the procedures followed were in accordance with the regulations of the relevant clinical research ethics committee and with those of the Code of Ethics of the World Medical Association (Declaration of Helsinki).

Confidentiality of data. The authors declare that no patient data appear in this article.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Conflict of interest

The authors declare no conflict of interest in the present study.

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