APPLICATION OF A PCR METHOD FOR THE DIAGNOSIS OF ULCERATIVE ENTERITIS: PRELIMINARY RESULTS

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Abstract
Ulcerative enteritis or "quail disease" is an acute clostridial infection of young birds reported in many avian species, chicken and turkey included. *Clostridium colinum* is the causative agent of ulcerative enteritis and because of the difficulties bound to the isolation and identification of this bacterium by means of classic bacteriological techniques, its detection appears very hard and the prevalence of this disease could be underestimated. To investigate the diffusion of *C. colinum* in enteric disease of birds, a recently developed PCR protocol was applied to 42 cultural broths previously inoculated with organs and intestinal samples collected from diseased subjects. PCR-positive broths were cultivated to attempt the isolation of *C. colinum*. Samples collected from positive birds were subjected to histological examinations. 4 birds (3 broilers chickens and 1 pigeon) resulted PCR-positive and, in one case, *C. colinum* was isolated. Gross and histological lesions of positive birds were compatible with those described in other ulcerative enteritis outbreaks. These preliminary results demonstrates that *C. colinum* is sporadically implicated in enteric diseases of broiler chickens (14.2%). In addition, the PCR assay proved to be an useful and reliable instrument to support the diagnosis of ulcerative enteritis and to facilitate the isolation of *C. colinum*.
Key words: diagnosis, ulcerative enteritis, *Clostridium colinum*, PCR

IMPIEGO DELLA PCR NELLA DIAGNOSI DELL'ENTERITE ULCERATIVA: RISULTATI PRELIMINARI

Riassunto
L'enterite ulcerativa o "quail disease" è il risultato di un'infezione acuta da *Clostridium colinum* che colpisce prevalentemente soggetti giovani e che è stata descritta in molte specie aviari, pollo e tacchino compresi. La diagnosi di tale patologia attraverso tecniche di microbiologia tradizionali risulta particolarmente indaginosa a causa dei lunghi tempi richiesti dall'esame batteriologico e dalla mancanza di terreni colturali selettivi. Con il presente studio si è voluta indagare l'utilità dell'applicazione di un protocollo in PCR nella diagnosi dell'enterite ulcerativa. A tale scopo una PCR specifica per *C. colinum* è stata applicata a 42 brodi colturali d'arricchimento precedentemente inoculati con materiale patologico prelevato da soggetti affetti da sindrome enterica. Successivamente gli organi e i campioni intestinali dei soggetti risultati positivi alla PCR, sono stati sottoposti ad accertamenti istopatolgici. 3 broiler e 1 piccione sono

risultati positivi alla PCR e in uno di questi casi è stato isolato *C. colinum.* Le lesioni anatomopatologiche e istologiche dei soggetti positivi erano compatibili con quelle descritte in altri episodi di enterite ulcerativa. Questi risultati dimostrano che la PCR è uno strumento utile ed affidabile per la diagnosi di enterite ulcerativa e può essere utilizzata come supporto all'isolamento di *C. colinum.* Inoltre, dai risultati preliminari, *C. colinum* appare sporadicamente (14,2 %) implicato nelle patologie enteriche che colpiscono i broiler in Italia.
Parole chiave: diagnosi, enterite ulcerativa, *Clostridium colinum*, PCR

Introduction

Ulcerative enteritis (UE) or "quail disease" is a severe bacterial disease induced by *Clostridium colinum* that affects various species of birds, including chickens and turkeys. An ulcerative-like disease associated with *C. perfringens* type A, has been recently reported in quails (*Colinus virginianus*) (Berkhoff, 1975; Shivaprasad *et al.,* 2008).
C. colinum is a spore forming obligate anaerobe which grows in 42 hours in enriched media. The lack of selective cultural media for *C. colinum* and the time consuming procedures required for its isolation, makes difficult the diagnosis of ulcerative enteritis by classic bacteriological techniques. To simplify the identification of *C. colinum* cultures and its detection in broths inoculated with organs and faeces, a species specific PCR protocol has been recently developed (Bano *et al.,* 2008). The present study reports the preliminary results arisen from the application of the cited PCR assay to cultural broths previously inoculated with organs and intestinal samples collected from birds with enteritis.

Materials and methods

Samples collection. 42 birds submitted in 2008 for post-mortem examination to the veterinary diagnostic laboratory of Treviso (Istituto Zooprofilattico Sperimentale delle Venezie), were included in the study on the base of enteric symptomatology or the presence of intestinal lesions. Portions of organs (liver and spleen) and intestine were collected from 7 quails (*Coturnix coturnix*), 21 chickens, 3 turkeys, 3 guinea fowls (Numida meleagridis), 3 pigeons (*Columba livia*), 1 pheasant (*Phasianus colchicus*), 1 swan, 1 parrot, 2 partridges (*Perdix perdix*).
Bacteriological examination. One gram of each sample was inoculated in an enrichment broth designed for spore forming anaerobes (Cooked Meat Medium, Difco) and the tubes were incubated at 37 °C in anaerobic chamber. After 42 hours of incubation, 1 ml of broth was processed by a species specific PCR for *C. colinum* and 10 µl of enriched broths were plated on Columbia Agar and in Perfringens Agar Base (Oxoid) containing 5% of sheep red blood cells. The plates were incubated for 42 hours at 37 °C. Strictly anaerobic colonies were subculturated on Columbia Agar containing 5% of sheep red blood cells and Colonies of Gram positive rods were processed by PCR for the identification of *C. colinum*.
Clostridium colinum PCR. 1 ml of enriched broths was centrifuged at 12.000 rcf for 3 minutes and DNA was extracted from the pellet by means of a commercially available kit (DNeasy Blood & Tissue Kit, Qiagen), according to manufacturer's instructions. The same kit was employed to extract DNA from pure bacterial cultures of anaerobe obligate Gram-positive rod shaped bacteria isolated from enriched broths tested positive at the PCR for *C. colinum*. To detect *C. colinum* a recently developed

species-specific PCR protocol was applied (Bano *et al.,* 2008). Major toxins (α, β1, ε, ι), enterotoxin , β2 toxin and NetB toxin encoding genes of *C. perfringens* isolates were searched by previously described PCR protocols (Drigo *et al.,* 2008; Keyburn *et al.,* 2008)

Histological examination. During the necropsy of chickens, portions of the same samples submitted for bacteriological examinations, were fixed in 10% neutral buffered formalin for 5 days. All tissues were then embedded in paraffin, sectioned at 4 μm, stained with haematoxylin and eosin and observed by light microscopy. Gram stain was also performed.

Parasitological and virological examination. Intestinal mucosa of all chickens was scraped in different districts and observed by optic microscope searching for protozoa and helminths. If hemorrhagic or necrotic intestinal lesions were present, parasitological examination was performed also directly on these lesions. The intestines were examined for viruses by negative stain electron microscopy by standard methods.

Results and discussion

The enteric lesions observed were classify as ulcerative enteritis (11/42), catarrhal or catarrhal-hemorrhagic enteritis (13/42) or necrotic enteritis (18/42). 3 chickens (14.2%) and 1 pigeon resulted positive at the PCR assay and *C. colinum* was isolated from the enriched broth inoculated with intestinal samples collected from the pigeon. Two of the three positive broiler chickens were 60 days old and came from the same farm. The third positive chicken was 40 days old. All PCR-positive chickens showed ulcerative enteritis and were negative for parasites and viruses but *C. perfringens* type A, *netB* negative was isolated. White foci of necrosis were disseminated in the hepatic parenchyma. At post-mortem examination the *C. colinum* positive pigeon showed hepatic focal necrosis and a severe haemorrhagic enteritis with abundant uncoagulable blood in the intestinal lumen. Whitish ulcers were visible through the serosa of the intestinal final tract and the lumen of the duodenum was enlarged by the presence of numerous helmints (ascarids). The histological examination of the three chickens revealed intestinal necrosis associated with lymphocyte infiltrations and the presence of Gram positive rod shaped bacterial aggregates. A lymphoid hyperplasia with a "starry sky" aspect and general congestion was observed in the spleen. In the liver, multifocal necroses of the hepatocytes associated with Gram positive rod shaped bacterial aggregates were detected. Bacterial aggregates were present also in the lumen of hepatic vessels and sinusoids.

These preliminary results demonstrates that *C. colinum* is sporadically involved in enteric diseases of broiler chickens in Italy even if sample size should be increased to establish more accurately the authentic diffusion of this micro-organism in diseased population. The PCR detected *C. colinum* DNA in 4 of 11 birds with lesions ascribable to ulcerative enteritis. The negativity of the other 7 subjects could be due to the implication of different intestinal pathogens or to the sensitivity of the PCR assay. The PCR results were sustained also by the histological detection of bacterial aggregates with morphological and Gram-stained characteristics referable to *C. colinum* in parenchymatous organs. *C. perfringens* strains were isolated only from the intestines and all strains belonged to toxin-type A (α-toxin coding gene positive) which is considered an usual finding also in healthy birds. The isolation of *C. colinum* resulted very hard because colonies were visible in 48 hours while the overgrowth of contaminants was

early. For this reason the develop of selective media should be take in consideration to support future investigations on *C. colinum.*

Conclusions

In conclusion *C. colinum* is sporadically implicated in enteric diseases of broiler chickens in Italy and the PCR assay proved to be an useful and reliable instrument to support the diagnosis of ulcerative enteritis and to facilitate the isolation of *C. colinum.*

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