A dead puppy was delivered to the laboratory for anatomo-pathological, virological and bacteriological examinations. The presence of canine parvovirus (CPV) was demonstrated by PCR in internal organs of the puppy. Bacteriological examinations proved that *E. coli* in pure culture was isolated from the small intestine of the dog. The isolate possessed the *estI* gene encoding heat-stable enterotoxin I (STI) as determined by the presence of the 166 bp PCR amplicon. The *E. coli* bacteria analysed were negative for all other virulence marker genes as well for fimbrial antigens tested in the study. To our knowledge, this is the first report in Poland describing the death of a puppy due to co-infection with canine parvovirus and enterotoxigenic STI-positive *E. coli* bacteria, with the characteristics of its virulence marker genes.

Key words: puppy, canine parvovirus, enterotoxigenic *Escherichia coli*, PCR.

Parvovirus infections in dogs are often reported in Poland, despite the wide use of vaccines. The disease is more commonly observed in homeless dogs and animals kept in kennels. Canine parvovirus (CPV) causes severe disease and clinical symptoms depending on the age, immunological status and feeding condition of animals as well as the antigenic type of circulating virus strains. The following symptoms of the disease are the most frequently observed: apathy, diarrhoea (often with the blood), vomiting and dehydration.

There is little information available concerning *Escherichia coli* infections in dogs. It has been documented that some strains of enterotoxigenic (ETEC) and enteropathogenic (EPEC) groups were clearly associated with gastro-intestinal disease in young dogs (3). Most canine ETEC strains were found to express heat-stable I (STI) enterotoxin but sometimes the strains were also positive for STII toxin (3). On the other hand, ETEC producing heat-labile enterotoxin LTI were not found in dogs (5, 11).
The aim of the presented studies was to confirm the canine parvovirus and enterotoxigenic STI-positive *E. coli* bacteria, as the cause of a fatal infection of puppies in a kennel.

**Material and Methods**

**Clinical findings.** The owner of a dog kennel observed a fatal disease among newborn German Shepherd puppies in different seasons of the year. In September 2002 he reported that during 5 weeks after birth, 6 out of 9 puppies demonstrated apathy, vomiting, watery diarrhoea and dehydration resulting in death of animals. Puppies were dewormed twice with pyrantel embonate (Pyrantel, PFO Vetos-Farma) in a dose of 15 mg/kg of body weight. The animals were not vaccinated against any virus infection. The bitch was vaccinated against distemper, adenovirus and parvovirus infections only once as a puppy. After the demonstration of the first clinical symptoms, all the puppies were treated with amoxicillin (Betamox L.A., Norbrook Laboratories) intramuscularly in a dose of 1 ml/10 kg of body weight and subcutaneously with electrolyte solutions, but the applied therapy was not successful. All the animals with clinical symptoms died between 5 and 10 d after the onset of the disease. The clinical symptoms as well as the information obtained from the dog owner suggested the infection of the puppy with parvovirus or canine adenovirus type 1 (CAV-1).

**Reference strains of viruses and bacteria used in the study.** The PD-14 reference canine parvovirus and adenovirus type 1 (CAV-1) strains belonging to the virus collection of the Department of Carnivores and Fur Animal Diseases were used as positive controls in the polymerase chain reaction (PCR). The CPV was propagated in feline lung cell line (Fc) and CAV-1 in dog kidney cell line (MDCK). The following *E. coli* reference strains were used as PCR controls: H10407 (*eltI*, *estI*, *astA*), 215/O26 (*estII*), B2 (*stx1*, *stx2*, *eaeA*, *ehlyA*), 214/O93 (*cnf1*), 214/O92 (*cnf2*), 491 (F40), 1547 (F5), 1500 (F6), 1540 (F17, F41), and C600 (K-12). The strains were described previously (12, 13, 14, 15, 16).

**Samples used for the studies.** The following samples of internal organs of the puppy were used for the study: liver, spleen, stomach, duodenum and jejunum. The samples were frozen and thawed. For virological examinations ten per cent homogenates of internal organs were prepared in PBS, centrifuged at 13 000 rpm for 15 min at 4°C and immediately used for PCR.

For the bacteriological examination, a swab from the small intestinal mucosa was taken and inoculated on blood and MacConkey agar plates. The bacteria (5 colonies from each plate) were identified as *E. coli* using the API 20E biochemical system (bioMerieux). The isolates were then tested for F4, F5, F6, F17, and F41 fimbrial antigens using the slide agglutination test as described previously (16).

**DNA isolation and polymerase chain reaction.** The isolation of viral DNA was done by the use of commercially available QIAamp DNA Mini Kit (Qiagen) according to the instruction of the producer. PCR was carried out with primers designated IW-1 and IW-3 (8) enabling to obtain the 583 bp fragment within the CPV VP2 capsid protein gene (EMBL Z 46651). For the detection of CAV-1 primers
designated CAV-1a and CAV-1b described by Kiss et al. (6) were used. They enabled to obtain the 301 bp fragment within the hexon protein gene of the virus. PCR parameters were described previously (9).

For the bacterial PCR, one *E. coli* colony was suspended in 25 µl of double-distilled water and heated at 99°C for 10 min. Then, the suspension was centrifuged (13 000 g, 1 min) to pellet the cellular debris and the supernatant was used as DNA template. The following *E. coli* virulence marker genes were tested: *eltI* (LTI enterotoxin), *estI* and *estII* (STI and STII enterotoxins, respectively), *stx1* and *stx2* of Shiga toxins 1 and 2, respectively, *eaeA* (intimin), *cnfI* and *cnfII* of cytotoxic necrotizing factor 1 and 2, respectively, and *astA* of enteroaggregative *E. coli* heat-stable enterotoxin 1 (EAST1) as described previously (12, 14, 15, 16).

**Electrophoretical analysis of PCR amplicons.** Ten µl of the PCR products were investigated using electrophoresis in 1.7% agarose gel in 1xTBE buffer (90 mM Tris, 90 mM boric acid, 2 mM EDTA), with constant voltage 100 V (5V/cm) for 90 min. The gel was stained with ethidium bromide (1 µg/ml) and visualized under the UV light (Hoefer Scientific Instruments). The size of amplicons was compared with pUC Mix Marker (Fermentas).

**Results**

The following pathological changes during post mortem examinations were observed: petechial congestion of small intestine mucosa enlargement mesenterial lymph nodes with the presence of petechiae. All the samples of the internal organs of the puppy were submitted to PCR. The presence of the 583 bp amplicon was demonstrated in the spleen, stomach, duodenum and small intestine confirming the infection with CPV (Fig. 1). An amplicon specific for CAV-1 was not detected in any samples tested.

Bacteriological examinations proved that *E. coli* in pure culture was isolated from the small intestine. These isolates possessed the *estI* gene encoding heat-stable enterotoxin I (STI) as determined by the presence of the 166 bp PCR amplicon in all bacterial colonies tested (Fig. 1, lane 4). The *E. coli* bacteria analysed were negative for all other virulence marker genes as well for fimbral antigens tested in the study.

**Discussion**

Parvovirus infection in dogs was first recognised in 1978 in the United States of America (1, 4). During one year CPV caused pandemics and illness among puppies and adult dogs (2, 10, 17). The isolated virus was called CPV-2. New antigenic types of the virus: CPV 2a and CPV 2b have been observed since then. The extent of antigenic changes among Polish isolates of canine parvovirus between 1982 – 1993 was the aim of studies performed by Mizak and Plucienniczak (7). The authors confirmed the presence of two antigenic types of CPV (CPV 2 and CPV 2a) in the dog population in Poland. Similar results were obtained by Rypula et al. (19).
The vaccination is the only efficient method to protect dogs against natural infection with canine parvovirus. All commercial vaccines available on the market efficiently protect dogs against CPV infection, but the immunization of the puppies performed during the immunity gap is the cause of vaccination failures.

![Agarose gel electrophoresis of PCR products amplified from DNA of CPV (lanes 1-3) and E. coli STI (lanes 4 and 5). Lane 1 - DNA isolated from spleen, lane 2 - from stomach, lane 3 - from small intestine, lane 4 - E. coli isolated from small intestine, lane 5 - estI positive E. coli control strain. M - pUC Mix Marker.](image)

Fig. 1. Agarose gel electrophoresis of PCR products amplified from DNA of CPV (lanes 1-3) and E. coli STI (lanes 4 and 5). Lane 1 - DNA isolated from spleen, lane 2 - from stomach, lane 3 - from small intestine, lane 4 - E. coli isolated from small intestine, lane 5 - estI positive E. coli control strain. M - pUC Mix Marker.

In this reported case, the lack of the proper vaccination scheme of the bitch might have resulted in the lack of specific antibodies in puppies and made them susceptible for parvovirus infection. Primary virus infection is often complicated by bacteria including E. coli. The inappropriate use of antibiotics resulted in the selection of resistant E. coli strains. ETEC were isolated as diarrhoeal pathogens from young dogs in several countries (3, 5, 11, 18, 20). However, to our knowledge, this is the first report in Poland describing the death of the puppy due to co-infection with canine parvovirus and enterotoxigenic STI-positive E. coli bacteria, with the characteristics of its virulence marker genes.

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