The antimicrobial susceptibility and virulence factors of *Bacillus anthracis* strains isolated in Croatia

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ABSTRACT: *Bacillus anthracis* can infect both livestock and humans. The importance of the treatment of the disease in humans has been underscored by the bioterrorism events of 2001 in the United States. The presence of *PA* and *B/C* genes (pX01 and pX02 plasmids) as well as susceptibility to several antimicrobial substances was determined in 11 strains of *Bacillus anthracis* isolated during two recent epizooties of anthrax which occurred in Croatia in 2002 among sheep and in 2006/2007 in cattle. The pX01 plasmid was observed in all of the examined strains, including vaccinal Sterne strains. However, the pX02 plasmid was detected in only eight out of eleven examined field strains of *Bacillus anthracis* while in vaccinal strains it was not detected at. Determination of MIC's revealed susceptibility to amoxicillin, amoxicillin with clavulanic acid, ciprofloxacin, gentamicin and tetracycline. All strains were resistant to sulfamethoxazole with trimethoprim and cefotaxime.

Keywords: *Bacillus anthracis*; antimicrobial susceptibility; virulence factors

Bacillus anthracis is a gram-positive spore-forming bacterium that can infect both livestock and humans. Infections of humans usually happen through direct contact with infected animals or contaminated animal products. The cutaneous form of the disease is predominant in human cases. The inhalation route of infection is rare and always leads to death (Friedlander et al., 1993). The virulence mechanism of virulent strains stems from the secretion of lethal factor and oedema factor toxins along with a spore-forming unit known as the protective antigen (Wattiau et al., 2008).

Antibiotic therapy in humans is important. The importance of treatment of the disease in humans has been underscored by the bioterrorism events of 2001 in the United States. The Center for Disease Control recommends ciprofloxacin, penicillin and doxycycline for the treatment of human cases and for prophylactic use after exposure and ciprofloxacin has been the drug of choice for prophylactic treatment after exposure to the spores (Mohammed et al., 2002). Penicillin, traditionally the drug of choice for treatment is still recommended in other parts of the world despite reports of resistance against penicillin (Coker et al., 2002). Streptomycin, gentamicin and chloramphenicol have also been recommended until recently (Mohammed et al., 2002).

Infection is rare both in humans and in animals in Croatia due to systematic immunoprophylaxis of cows, sheep, goats and horses in enzootic areas where outbreaks of anthrax had occurred in the past decades. A total of 27 sporadic cases have occurred over the last 30 years. The disease was confirmed in cows (14 cases), sheep (seven cases), goat (four cases) and horses (three cases). Disease progression is characterised by the absence of disease in some periods. Therefore, within the last three decades the disease was recorded in 17 years while in 13 years it was not detected (1981–1983, 1992–1996, 1998–2001 and 2008–2010). The spatial distribution of cases is characterised by a concentration of sporadic cases around the City of Sinj where 14 cases were recorded over several years. Furthermore two

outbreaks of anthrax were recorded during the mentioned time. The first outbreak occurred in 2002 near Jasenovac when 11 sheep died in a flock. Samples from five sheep were subjected to laboratory examination. Another outbreak occurred near Sunja over the winter of 2006/2007 when 11 cows were affected (Anonymous 1980–2010). Both epizooties affected a relatively small number of animals, due to the fact that control measures were implemented immediately after diagnosis (Habrun et al., 2009).

The locations of outbreaks and sporadic cases are shown in the map in Figure 1. The settlements of Jasenovac and Sunja are situated along the Sava River, while most sporadic cases occurred near city of Sinj. Other cases of outbreaks of anthrax have occurred around Donji Lapac, Obrovac, Gračac, Otočac. Only two cases were recorded in northwest Croatia.

The aim of this study was to isolate *B. anthracis*, subject isolates to PCR detection of *PA* and *B/C* genes and to determine the antimicrobial susceptibility of *B. anthracis* isolated from animals which died over the course of two outbreaks.

MATERIAL AND METHODS

Isolation of agents

Two spleens and three years with surrounding tissue were sampled and tested during the 2002 outbreak. The spleens were sampled and tested at the onset of the disease, after two autopsies had been conducted. After the diagnosis, there were no more autopsies; further samples consisted of ear with surrounding tissue. During the 2006/2007 outbreak a total of 11 samples were examined (surrounding tissue of the ear only). Tissue samples were homogenised and inoculated onto blood agar with 5% defibrinated sheep blood (Quinn et al., 1994).

Figure 1. Geografic distribution of anthrax outbreaks (red images) and sporadic cases (black images) in Croatia during last 30 years

After incubation at 37 ± 1 °C for 18–24 hours, plates were examined and colonies were morphologically inspected. Colonies which showed specific morphology (non-haemolytic, or which had a small haemolysis zone with curly endings forming medusa like shapes) were sub-cultured onto blood agar for further identification (Quinn et al., 1994; Anonymous, 2008).

Determination of capsule formation

Pure culture was further inoculated onto Columbia agar plates with the addition of 0.7% sodium bicarbonate and incubated in 5% CO_2 (Genbox CO_2, BioMerieux). After 18–24 hours of incubation, "S"-formed colonies were observed due to capsule formation.

Submitted samples were smeared directly and stained using the M'Fadyean reaction (polychrome methylene blue staining) for evidence of capsule formation.

Determination of motility

Suspected colonies were inoculated into tripticase soy broth and incubated at 37 ± 1°C for 24 hours for detection of motility. Motility was examined in the dark field of the microscope to differentiate non-motile *B. anthracis* from motile *B. cereus* strains.

Antimicrobial disc susceptibility testing

Susceptibility testing was performed to the following antimicrobial agents using the disc diffusion method according to CLSI (2008) recommendations (M31-A3): penicillin (10 IU), ceftazidime (30 µg), cefotaxime (30 µg), ceftriaxone (30 µg) and cefoperazone (75 µg). Susceptibility to penicillin was determined in order to differentiate *B. anthracis* from *B. cereus* (Quinn et al., 1994). Susceptibility to three cephalosporins was determined after a high resistance level had been observed in the determination of the MIC to cefotaxime.

According to the CLSI, Mueller-Hinton agar is to be used as a culture medium (Merck 1.05435). *Escherichia coli* ATCC 25922, and *Staphylococcus aureus* ATCC 25923 were used as control strains. Zone interpretations for *B. anthracis* have not been established by CLSI. Hence we used the interpretative diameter zone for non-fastidious microorganisms (cephalosporins) and for staphylococci (penicillin). Depending on the zone of inhibition results were interpreted as susceptible, intermediary susceptible and resistant, as recommended by CLSI (M100 S-17) (2007).

All collected isolates were stored in Luria-Bartrani broth with glycerine at –70 °C before MIC determination and PCR examination. Determination of susceptibility to cephalosporins with the disc diffusion method, determination of MIC's and PCR were done on 11 strains, five of which were isolated from sheep during the 2002 outbreak and six from cattle during the 2006/07 outbreak.

MIC determination

The minimum inhibitory concentration (MIC) was determined by the use of the *E*-test (AB Biodisk, Sweden). The test is an expansion of the disc diffusion method with the same agar and inoculum preparation. The antibiotic content of the strip is graded, and the concentration is printed linearly along the strip. The test was performed according to the manufacturer's instructions. The MIC was determined for the following antimicrobials: amoxicillin, amoxicillin + clavulanic acid, cefotaxime, ciprofloxacin, gentamicin, spectinomycin, streptomycin, tetracycline and sulfamethoxazole + trimethoprim.

Antimicrobial agent concentrations ranged from 0.002 to 32 µg/ml (ciprofloxacin, and trimethoprim + sulfamethoxazole), from 0.016 to 256 µg/ml (amoxicillin, amoxicillin + clavulanic acid, cefotaxime, gentamicin, and tetracycline), and from 0.064 to 1024 µg/ml (spectinomycin and streptomycin), while *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 were used as control strains.

Confirmation of virulence using the polymerase chain reaction (PCR)

Confirmation of virulence was carried out by PCR using the primers PA5, PA8, 1234 and 1301. The expected amplification product size for the *PA* gene fragment (plasmid pX01) is 596 bp and for the capsule *B* and *C* gene fragment (plasmid pX02) it is 846 bp (Ramisse et al., 1996).

Pellets from bacterial cultures were resuspended in 50 µl of Q water (Sigma, Germany), heated to 99 °C

for 20 min and centrifuged at 14 000 g for 1 min. The resulting supernatant was used as DNA template for the PCR. The PCR for the identification of pX01 and pX02 plasmids was carried out in a 50 µl reaction mixture containing 0.25 µl of AmpliTaq DNA Polymerase (5 IU/µl, Applied Biosystems, USA), 4 µl of dNTP (10mM, Applied Biosystems, USA), 5 µl of 10 × PCR Buffer II (Applied Biosystems, USA), 3 µl of $MgCl_2$ (25mM, Applied Biosystems, USA), 33.75 µl of water (Qiagen, Germany), 0.5 µl of forward and 0.5 µl of reverse primer (160µM, Invitrogen, Scotland) and 3 µl of DNA template. Amplifications were carried out in a GeneAmp® PCR System 2700 (Applied Biosystems, USA) with a polymerase activation step (95 °C/15 min), followed by 40 cycles of denaturation (95 °C/30 s), annealing (55 °C/30 s) and extension (72 °C/30 s, and a final extension step (72 °C/10 min). The primers PA 5 (TCCTAACACTAACGAAGTCG) and PA 8 (GAGGTAGAAGGATATACGGT) were used to confirm the presence of the gene coding for the protective antigen of the plasmid pX01. The primers 1234 (CTGAGCCATTAATCGATATG) and 1301 (TCCCACTTACGTAATCTGAG) were used to confirm the presence of the genes *B* and *C* of the capsule region of the plasmid pX02. The expected amplification product size for the plasmid pX01 is 596 bp and for the plasmid pX02 it is 846 bp. Amplification products were separated in 2% agarose gel and stained by ethidium bromide. Visualization was achieved with a UV transluminator and the BioCapt camera Document System (Vilbert Lourmat, France).

RESULTS

Isolation of agents

During the 2002 outbreak *B. anthracis* was isolated from all five examined samples (two spleen samples and three ear tissue samples). All isolated strains were subjected to further examination. During the 2006/07 outbreak *B. anthracis* was isolated from 11 ear tissue samples. However, only six randomly selected strains were selected for further examination. Rough, non-haemolytic colonies of *B. anthracis* were isolated from all tissue samples (either spleen or ear tissue). All isolated strains formed capsules when cultivated on Columbia agar with 0.7% Na bicarbonate in 5% CO_2. The isolates were non-motile and the colonies formed medusa head-like forms after 48 hours of incubation. All isolates were susceptible to penicillin based on the disc diffusion method (disc 10 IU).

Susceptibility testing

The results of susceptibility testing using the disc diffusion method and the results of MIC determination are presented in Tables 1 and 2, respectively. Although susceptibility testing was carried out on *B. anthracis* of sheep (five isolates) and bovine origin (six isolates) the observed differences cannot be linked to the origin.

Confirmation of virulence using the polymerase chain reaction (PCR)

PCR analysis was carried out on 14 bacterial samples l including 11 field isolates of *B. anthracis*, two vaccinal strains (Sterne) and a sample of a *B. cereus* strain that served as a negative control. The *PA* gene (plasmid pX01) was present in eight field strains as well as in vaccinal strains. All field strains contained the capsule *B* and *C* genes (plasmid pX02). There was no evidence of capsule *B*/*C* genes in vaccinal strains (Sterne). Expectedly there was no evidence of the *PA* gene or the capsule *B*/*C* genes in *B. cereus* ATCC 11778.

DISCUSSION

Determination of the susceptibility of *B. anthracis* is important in order to determine which therapy to use in humans. Antibiotic therapy in humans is very important for prevention after exposure and for treatment after infection (Center for Disease Control and Prevention, 2006).

Ruminants are usually affected with a peracute or acute form of disease. Severely ill animals are unlikely to recover but in the early stages, particularly when fever is detected before other signs, recovery can be anticipated if the correct treatment is provided (Radostits et al., 2000).

B. anthracis is usually susceptible to a broad range of antibiotics (Cavallo et al., 2002). MIC ranges, MIC_{50} and MIC_{90} values for our field isolates are shown in Table 2. These isolates were quite susceptible to amoxicillin, amoxicillin with clavulanic acid, ciprofloxacin, gentamicin and tetracycline *in*

Table 1. Antimicrobial susceptibility of *Bacillus anthracis* (n = 11) by disc diffusion method*

Antimicrobial agent	Susceptible	Intermediatery susceptible	Resistant
Penicillin	11	0	0
Ceftazidime	0	0	11
Cefotaxime	0	2	9
Ceftriaxone	2	9	0
Cefoperazone	11	0	0

*Results are expressed as number of 11 *Bacillus anthracis* isolates susceptible, intermediate/moderately susceptible and resistant to each antimicrobial agent

vitro. Good susceptibility to ciprofloxacin, gentamicin and tetracycline has also been described in France (Cavallo et al., 2002).

According to the results using the disc diffusion method, all isolates were susceptible to penicillin (Table 1). Cavallo et al. reported the resistance of 11.5% strains to penicillin and amoxicillin (Cavallo et al., 2002). In another study, 22 *B. anthracis* isolates were tested for susceptibility to 27 antimicrobial agents by agar dilution. All isolates were sensitive to penicillins and did not produce beta-lactamase (Doganay and Aydin, 1991). Although such resistance was not reported in Croatia, it should be taken into account at post-exposure prophylaxis.

Resistance to sulfamethoxazole with trimethoprim was determined in all isolates (Table 2), which is in compliance with the earlier results reported from France and from the Kruger National Park in South Africa (Odendal et al., 1991; Cavallo et al., 2002). These results support the claim that sulfamethoxazole with trimethoprim should not be used in anthrax prophylaxis or treatment in humans (Esel et al., 2003).

A high level of resistance to cefotaxime was determined (MIC_{50} > 256 mg/l) and for that reason we checked the susceptibility to cefotaxime and three other 3rd generation cephalosporins, namely, ceftazidime, ceftriaxone and cefoperazone, by the disc diffusion method. All isolates were susceptible to cefoperazone, two isolates were susceptible and nine moderately susceptible to ceftriaxone. Two isolates were moderately susceptible to cefotaxime according to the disc diffusion method and showed MIC's of 4 and 8 mg/l respectively. It has previously been determined that all isolates were resistant to a 3rd generation cephalosporin (ceftriaxone) (Cavallo et al., 2002). Also, Duganay and Aydien (1991) reported that all tested isolates (22) were susceptible to cefazolin, cephalotin, cephradine and cefoperazone, 19 isolates were resistant to cefuroxime, 18 to cefotaxime, 18 to ceftizoxime, 9 to ceftriaxone and 21 to ceftazidime. Ofloxacin and ciprofloxacin showed very good activity at MICs of 0.03–0.06 mg/l. Based on our results and the results of former studies 3rd generation cephalosporins can be taken into account for postexposure treatment and therapy of humans.

The determination of virulence factors using PCR showed that all isolates except for the vaccinal strains harboured the capsule *B* and *C* genes (pX02 plasmid) and that they formed a capsule (Sterne). The *PA* gene (plasmid pX01) was not found in isolates 1, 9 and 13, although they were isolated from animals that died from anthrax. This absence of the *PA* gene could be interpreted as indicating

Table 2. Detection of minimum inhibitory concentration (mg/l) in 11 isolates of *Bacillus anthracis*

Antimicrobial agent	MIC_{50}	MIC_{90}	MIC range
Amoxicillin	0.023	0.032	0.016–0.047
Amoxicillin + clavulanic acid	0.023	0.032	0.016–0.047
Cefotaxime	>256	>256	4.0–>256
Ciprofloxacin	0.064	0.094	0.047–0.125
Gentamicin	0.064	0.0125	0.032–0.125
Spectinomycin	12	16	12–32
Tetracycline	0.023	0.032	0.016–0.047
Sulfamethoxazole/trimethoprim	>256	>256	>256

that some strains of *B. anthracis* may not carry the pX01 or pX02 plasmid at all or that they lose these genes due to growth under laboratory conditions (Anonymous, 2008).

In conclusion, *B. anthracis* remains susceptible to many antibiotics, including amoxicillin, tetracycline and ciprofloxacin. The resistance of isolates to penicillin and amoxicillin in some earlier reports has shown that such antibiotics should not be used in human therapy without prior determination of susceptibility (Cavallo et al., 2002).

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