DÉNES ATTLA-LEHEL

Summary of the Ph-d thesis

STUDY REGARDING BACTERIAL SPECIES FROM GENUS BORDETELLA ISOLATED FROM ANIMALS AND THEIR IMPORTANCE IN THE VETERINARY PATHOLOGY

Scientific coordinator
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CLUJ-NAPOCA
2010
SUMMARY

The investigations conducted under the thesis „Study regarding bacterial species from genus Bordetella isolated from animals and their importance in the veterinary pathology” were performed between the period of 2004-2010 and had the following main objectives:

- Isolation of *Bordetella bronchiseptica* strains from different host species (pigs, dogs, cats, horses and rats), isolation of *Bordetella parapertussis* from sheeps and *Bordetella avium* from birds (hens, chickens and turkeys), testing a seminificative amount of samples;
- To conduct a carrier status study of these *Bordetella* species using bacteriological tests;
- Identification of *Bordetella* strain isolated from animals using bacteriological, bacterioscopical and biochimical tests;
- Observation regarding the impact of the *Bordetella* species on different naturally infected host species;
- Identification of adequate culture medias for isolation of different *Bordetella* species;
- Verification on the capacity to induce antibody synthesis after inoculation in rabbits and the capacity to induce a protective immune response in rats of an antigenic solution obtained from sonicated *Bordetella bronchiseptica* cultures;
- Verification regarding the possibility to use sonicated *Bordetella bronchiseptica* antigenic solutions in serological diagnosis of *Bordetella bronchiseptica* infection;
- To verify if a prolonged therapy with high dose of corticosteroids in rats can produce deep immunosupression; *Bordetella bronchiseptica* carrier rats were treated with prednisone and they were monitored for any signs of respiratory distress;
- Serological diagnosis of *Bordetella bronchiseptica* infection in pigs and dogs using slow agglutination test and double immunodiffusion test; we compared the sensitivity of these tests to establish if they can be used or not in current laboratory diagnostic of *Bordetella bronchiseptica* infection in pigs and dogs;
- To determine the inhibitory effect of some antibiotics on the isolated *Bordetella bronchiseptica* and *Bordetella parapertussis* strains;
- To performe test regarding the capacity of *Bordetella bronchiseptica* and *Bordetella parapertussis* to survive in different enviromental conditions, knowing that high enviromental resistance can show that the bacteria has an enviromental reservoir;
The thesis contains 301 pages and it is structured according to the current legal demands in two main parts: the first one entitled “Bibliographic studies”, contains 83 pages structured on 6 chapters an it represents 27.57% of the thesis. The second part “Personal research” has 218 pages structured on 8 chapters, representing 72.43%. The thesis contains 90 images and 22 charts, designed to help synthesize the results and to contribute to a better understanding of the practical aspects. The Bibliography contains 168 titles of literature from Romania or other countries.

The first part, entitled „Bibliographic studies” presents information about taxonomy and evolution of Bordetella species. Further in this part are described filogenetic relationships among Bordetella species and eco-biology of these bacteria. There are presented the methods of sample collection for isolation of bordetelleae, methods of isolation and identification of different species among genus Bordetella. Patogenesis, immunogenic structures and the patogenetic structures of these bacteria are described in an extended chapter. This part contains information about the immune response of the host species and in the end of this part is a chapter dedicated for the description of the clinical affections developed by host species after infection with different species of bordetella. This chapter also contains information about terapy and immunoprevention of these affections.

Genus Bordetella contains nine species, one of them are human patogen, others has implication in the veterinary pathology. Bordetella bronchiseptica is the most important animal pathogen. It has the capability to infect any mamallian species and it is involved at least in three well documented clinical affections: atrophic rhinitis in pigs, kennel cough in dogs and Upper Respiratory Tract infection in cats. Bordetella avium produces respiratory infections in birds and turkey bordetelosis is the most common affection produced by this bacteria. Bordetella parapertussis ov infects the respiratory tract of sheeps, enhancing the development of pneumonia due to Pasteurella multocida.

The second part „Personal research” contains 6 chapters, a general discussion of the results, final conclusions and bibliography.

In the seventh chapter, entitled „Aspects regarding isolation and identification of animal pathogen bacterial species among genus Bordetella” were presented and subdue to discussion the results obtained after sample collection from pigs, dogs, cats, horses, rats, sheeps and birds and sample submission to bacteriological tests. Description regarding sample collection, sample submission to bacteriological tests, insemination methods, and bordetelae identification are presented.

A total number of 543 samples were collected for isolation of B. bronchiseptica. The number of isolated strains was 58.

The number of samples collected from pigs was 302, from dogs was 123, from cats was 72, from horses was 26 and 12 samples were collected from rats.
In small animals sample collection was performed by nasal cavity washing and in large animals (large piglets, large dogs and horses) nasopharingial swabs were used. The two methods were combined in cases when animals presented nasal discharges.

From the 302 samples collected from pigs 21 strains of *B. bronchiseptica* were isolated, the percent of isolation was 6,953%. Nineteen strains of *B. bronchiseptica* were isolated from alive pigs and the number of sampled alive pigs was 259. Two strains were isolated from pneumonic lungs collected from weaned pigs, in these cases *B. bronchiseptica* was isolated in pure culture. Only one strain of *B. bronchiseptica* was isolated from the 37 samples collected from healthy neonate piglets, isolation percentage in this case being only 2,702%. In case of neonate piglets presenting respiratory distress (11 samples collected) isolation succeeded in 5 cases, leading to the highest isolation percentage: 36,363%. The overall results in neonate pigs showed 6 isolated strains from 48 samples (isolation percentage: 10,416%). In case of the 208 samples collected from weaned piglets the number of isolated strains were 13, with an isolation percentage of 6,25% (table 1). Only three samples were collected from weaned pigs presenting signs of respiratory distress, but even in this cases a strain of *B. bronchiseptica* was isolated. In this case *B. bronchiseptica* was isolated from a pneumonic piglet and the bacteriological exam revealed a great number of bordetella in the nasal mucus (image 1), probably the pneumonia was caused by *B. bronchiseptica*. This piglet was fully recovered after a parenteral treatment with enrofloxacin.

Fourtyfree samples were collected from pneumonic pig lungs. Two strains of *B. bronchiseptica* were recovered, isolation of this strains were made in pure culture on MacConkey agar. One sample was collected from a 4 months old pig and the other from a 3 months old pig, both of them died because of a severe bronchopneumonia.
The results of the cultural exam for samples collected from pigs

Table nr.1

<table>
<thead>
<tr>
<th>Type and origin of the examined samples collected from pigs</th>
<th>Total samples number</th>
<th>Positive samples</th>
<th>Negative samples</th>
<th>Isolation percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alive pigs</td>
<td>259</td>
<td>19</td>
<td>240</td>
<td>7,334%</td>
</tr>
<tr>
<td>Piglets with age under 6 weeks without respiratory distress</td>
<td>37</td>
<td>1</td>
<td>36</td>
<td>2,702%</td>
</tr>
<tr>
<td>Piglets with age under 6 weeks with signs of respiratory distress</td>
<td>11</td>
<td>4</td>
<td>7</td>
<td>36,363%</td>
</tr>
<tr>
<td>Weaned pigs without respiratory distress</td>
<td>208</td>
<td>13</td>
<td>195</td>
<td>6,250%</td>
</tr>
<tr>
<td>Weaned pigs with signs of respiratory distress</td>
<td>3</td>
<td>1</td>
<td>2</td>
<td>33,333%</td>
</tr>
<tr>
<td>Pneumonic lungs</td>
<td>43</td>
<td>2</td>
<td>41</td>
<td>4,651%</td>
</tr>
<tr>
<td>Total examined samples</td>
<td>302</td>
<td>21</td>
<td>281</td>
<td>6,953%</td>
</tr>
</tbody>
</table>

Regardless of the culture media isolation of *B. bronchiseptica* from alive pigs is very difficult because samples are always contaminated with *E. coli*. This bacteria acidifies the culture medias and made impossible the development of *B. bronchiseptica* colonies, so the bacteriological test lacks sensitivity. This is the reason why bacteriological exam is not adequate for *B. bronchiseptica* carrier status study in pigs (image 2).
Image 1, Strain 19 of *B. bronchiseptica* isolated on MacConkey agar

Image 2, Overgrowth of *E. coli* (yellow colonies), acid reaction in the culture media; only a few colonies of non-fermenting bacteria developed colonies in these samples (green colonies)

We can conclude that in neonate piglets infection with *B. bronchiseptica* has the highest chance to develop clinical symptoms while older animals tend to be carriers of the bacteria. *B. bronchiseptica* is associated with atrophic rhinitis, but during this research we have never observed a single pig with characteristic symptoms of this clinical condition. Even if *B. bronchiseptica* was isolated from many pigs we believe that in pig farms the abuse of the large
spectrum antibiotics prevents the extensions of the lesions due to _B. bronchiseptica_.

Samples were collected from 123 dogs. Sixty samples were collected from dissected animals and 63 samples came from alive dogs (table 2). Alive dogs were also submitted to blood sample collection for serological diagnosis of _B. bronchiseptica_ infection.

The diversity of the examined samples for _Bordetella bronchiseptica_ strains isolation

<table>
<thead>
<tr>
<th>Source of samples collected from dogs</th>
<th>Alive dogs examined in the Emergency Care Unit</th>
<th>Necropsied dogs</th>
<th>Total sample number</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dogs presenting respiratory tract affections with age belowe one year</td>
<td>19</td>
<td>22</td>
<td>41</td>
</tr>
<tr>
<td>Dogs presenting respiratory tract affections with age above one year</td>
<td>13</td>
<td>9</td>
<td>22</td>
</tr>
<tr>
<td>Dogs without respiratory tract affections with age belowe one year</td>
<td>21</td>
<td>17</td>
<td>38</td>
</tr>
<tr>
<td>Dogs presenting respiratory tract affections with age above one year</td>
<td>10</td>
<td>12</td>
<td>22</td>
</tr>
<tr>
<td>Total sample number</td>
<td>63</td>
<td>60</td>
<td>123</td>
</tr>
</tbody>
</table>

Isolation of _B. bronchiseptica_ from dogs was sucessful in 12 cases, isolation percentage regarding the number of the overall samles was 9,756%. In case of the alive dogs 8 strains of _Bordetella bronchiseptica_ were isolated, isolation percentage in this group was 12,698%. Four strains were isolated from dogs presenting typical simptoms of kennel cough. These dogs were under 12 months of age, presenting tracheobronchitis, dry cough, paroxistical cough when the trachea was examined, slight bilateral nasal discharge. Thei did not presented fever and their general condition was good. In three cases the animals were recovered completely in 9-12 days after treatement with Synulox and codeine. One dog developed distemper after 2 days of evolution of the kennel cough and it was euthanasied. In these cases _B. bronchiseptica_ was isolated almost in pure culture (image 3).
Other four *Bordetella bronchiseptica* strains were isolated from healthy dogs that exceeded the age of 10 months. In case of the necropsied dogs also four strains of *Bordetella bronchiseptica* were isolated. Three of them were isolated from young dogs (aged between 4-6 months) that died of distemper and their lungs were invaded by *Bordetella bronchiseptica*. In one occasion isolation was made from a mucus sample collected from a major bronchiae.

Isolation and identification of *Bordetella bronchiseptica* from samples collected from dogs is easy.

The studied cats attended a shelter or they lived a period in this place. A few cats gave birth at this shelter. In the period of the study (May 2005-August 2009) a total of 39 kitten that lived at least 3 weeks were born here. The total number of animals included in this study was 71 young cats and the number of isolated *B. bronchiseptica* strains was 19. Isolation percent was 26.761%. In the period of the study 13 cats born at the shelter developed typical symptoms of Upper Respiratory Tract Disease. Out of thirteen survived only 7, the mortality among these kittens was 46.153%.

Isolation of *Bordetella bronchiseptica* from cats is relatively easy because sample contaminants grows slowly on MacConkey agar, allowing *B. bronchiseptica* to form typical colonies on this culture media.

Samples were taken from 26 horses and six strains of *B. bronchiseptica* were isolated, the isolation percent reaching 23.076%. All strains were isolated from the same place, from a small horseclub in Miercurea Nirajului. Nine samples were taken here, so the isolation percentage in these animals reached
66.66%. In spite of the fact that these horses were *B. bronchiseptica* carriers, they did not present any clinical signs of infection.

Isolation of *B. bronchiseptica* in horses with the use of MacConkey agar is very laborious because sample contaminants are slow growing too and they form colonies with morphology similar to *B. bronchiseptica*.

In case of the twelve rats taken in study samples were *B. bronchiseptica* free.

During isolation and identification of *B. bronchiseptica* observation were made regarding cell morphology, the colonies morphology on MacConkey agar (image 4), on modified Smith-Baskerville agar (image 5), on sheep blood agar (image 6) on Istrati-Meitert culture media (image 7) and liquid nutrient broth. Cultivated on MacConkey agar or on blood agar the colonies of *B. bronchiseptica* develops a typical morphology after an incubation of 2 day at 37°C. The colonies are 2 mm in diameter, pink with a discoloration of the adjacent medium in amber like colour on MacConkey agar and colonies are slight gray coloured on blood agar with the presence of a hemolitic zone under the colonies. On modified Smith-Baskerville culture media after an incubation period of 48 hours the colonies of *B. bronchiseptica* can be green or blue, depending on the capacity of the strain to have a slight or powerfull alcane reaction on this culture media. On Istrati-Meitert agar *B. bronchiseptica* manages to form visible colonies after 24 hours of incubation. These colonies are small (1-1.5 mm in diameter) and usually are blue, but they can be gray too. Agglomerated colonies have a greenish-greey colour.

All isolated *B. bronchiseptica* strains had the same biochemical profile.

Gramm staining of *B. bronchiseptica* revealed small (0.2-0.3µm /0.4-1.5 µm) rod shaped bacterial cells coloured in red, very similar to *B. parapertussis* cells (image 8).
Image 4, Pure culture of *Bordetella bronchiseptica* on MacConkey agar after an incubation of 48 hours at 37°C;

Image 5, Pure culture of *Bordetella bronchiseptica* on MacConkey agar after an incubation of 48 hours at 37°C;
Even after 263 samples taken and carefully examined *Bordetella avium* could not be isolated from birds. It seems that birds population taken in study were *B. avium* free.
*Bordetella parapertussis* was isolated from sheeps. A total number of 121 samples were tested but only two strains were isolated (isolation percent was 1,653%). The bacteriological positive sheeps were only carrier of the bacteria, they did not showed any signs of respiratory distress.

*Bordetella parapertussis* is a very slow growing bacteria so it’s isolation can be performed only on highly selective culture medias. Moredum Bordetella Medium proved to be an excelent culture media for isolation and identification of this bacteria. *B. parapertussis* develops little colonies on this culture media after an aerobic incubation period of 3 days at 37°C. These colonies are surrounded by a wide zone of complete haemolisis. After an additional two days of incubatin the colonies reaches at 2 mm in diameter. Identification of *B. parapertussis* is easy. Cultural aspects on Moredum Bordetella Medium (image nr.9) and in liqid culture media (image nr.10) corelated with the slow growth makes easy to identify this bacterial species.

*B. parapertussis* cells looks like *Bordetella bronchiseptica* cells after Gram staining. They are rod shaped Gram negative small(0,2-0,3µm /0,4-1,5 µm) bacteria (image 8).

Image 8, *Bordetella bronchiseptica* after Gram staining  (x 1600)
Image 9, *Bordetella parapertussis*<sub>ov</sub>, culture on Moredum Bordetella Medium

Image 10, Three days culture of *Bordetella parapertussis*<sub>ov</sub> in liquid culture media
In the eight chapter entitled „Researches regarding anti-Bordetella bronchiseptica antibodies production by rabbits hyperimmunisation” the main goal of the studies was to observe the capacity of disrupted and subcutaneously injected Bordetella bronchiseptica cells to induce a measurable humoral immune response. An another objective of the study was to determine if the obtained antigen solutions and hyperimmun mun serums are appropriate for laboratory use in serological detectin of the Bordetella bronchiseptica infection using double immunodiffusion test. After antigen inoculation (every time we used a higher quantity) the dynamic of antibody synthesis was observed and the use of the double immunodiffusion test allowed us to make observation regarding the minimal number of immunogenic structures that induced a humoral response after each inoculation of antigen solutions.

In this study we used four rabbits, a control rabbit injected with physiological saline and three rabbits used for antigen solution administration.

We used two antigen solutions, one prepared from a dog isolate of Bordetella bronchiseptica and another prepared from a pig isolate of Bordetella bronchiseptica. Isolates were grown on blood agar and suspended in physiological saline. These suspensions were used for bacterial desintegration using a DUS P 150 ultrasound desintegrator. Total desintegration of the bacterial cells was achieved after 90 minutes. The obtained solutions were centrifuged 30 minutes at 6000 rpm. The result was a clear, yellow-gray solution. Antigen solutions were diluted to a protein content of 2 mg/ml, formaldehyde was added to a final concentration of 0.5 %. These antigen solutions were used in rabbit inoculation and in double immunodiffusion tests.

Rabbits were inoculated three times. At first inoculation we used a volume of 1 ml antigen solutions, at second inoculation (14 days after first inoculation) the volume used was 2 ml, and the final inoculation (14 days after the second inoculation) was made with 3 ml of antigen solutions. The rabbits tolerated well the antigen solutions, without any local or general reactions. Blood samples were collected after 7 days in case of the first two inoculations and after 14, 28 and 45 days after the third inoculation (Table nr.3).

<table>
<thead>
<tr>
<th>Inoculation number</th>
<th>Dosage (ml)</th>
<th>Inoculation path</th>
<th>Inoculation day</th>
<th>The day of blood collection</th>
<th>Collection number</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>1</td>
<td>s.c.</td>
<td>0</td>
<td>-7</td>
<td>1</td>
</tr>
<tr>
<td>II</td>
<td>2</td>
<td>s.c.</td>
<td>14</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>III</td>
<td>3</td>
<td>s.c.</td>
<td>28</td>
<td>42</td>
<td>4</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>56</td>
<td>42</td>
<td>5</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>73</td>
<td>6</td>
</tr>
</tbody>
</table>
Antibody production during the hyperimmunization was monitored with double immunodiffusion in agar gel, slide agglutination test and slow agglutination test. Agglutination tests needed a cellular antigen, obtained with formaldehyde inactivation of a *Bordetella bronchiseptica* suspension.

Antigens obtained from desintegrated *Bordetella bronchiseptica* cells were highly immunogenic. Anti-*Bordetella bronchiseptica* antibodies could be detected in the rabbit’s serum even in 7 days after the first antigen administration using double immunodiffusion and slow agglutination tests. At this moment the double immunodiffusion test showed a single precipitation line in all three cases and anti-*Bordetella bronchiseptica* antibody titre determined with slow agglutination tests varied between 1/10 and 1/40 (image 10 and 11). During the hyperimmunization protocol the humoral immune responses of the rabbits grew in intensity and diversity, but in all three cases the immune response followed the same pattern. After the second antigen administration slide agglutination tests turned positive. The highest level of anti-*Bordetella bronchiseptica* antibodies was achieved after 28 days following the third antigen administration. At this moment in the double immunodiffusion test could be observed 4 precipitation lines when sera obtained from the two rabbits inoculated with the dog isolate of *Bordetella bronchiseptica* were used. In case of the rabbit inoculated with antigen solution obtained from the pig isolate of *Bordetella bronchiseptica* the number of observed precipitation lines were five (image 12).

The antibody titres at this moment were between 1/1280 and 1/2560. Double immunodiffusion test allowed to identify two immunogenetic components of the antigen solutions: the filamentous haemagglutinin and lipopolisacharides. This technique also showed that there are differences between the antigenic structures of the two isolate of *Bordetella bronchiseptica* so this bacterial species presents differences regarding their immunogenetic structures. Laboratory tests showed that antigen solutions prepared in our laboratory as well the sera obtained from the rabbits can be used in the serological diagnosis of *Bordetella bronchiseptica* infection.
Image 10, Positive slow agglutination test: the agglutinin pattern was judged as positive when only definite granules of antigen were seen in a layer covering the bottom of the wells;

Image 11, Negative slow agglutination test: the agglutinin pattern was judged as negative when a button shaped deposit could be observed in the bottom of the wells;

Image 12, Double immunodiffusion in agar gel with use of sera samples obtained from rabbits after 28 days from the third antigen inoculation and antigen solution prepared by use of pig isolate of *Bordetella bronchiseptica* (A: well containing antigen solution; 1: wells containing serum obtained from rabbit number 1; 2: wells containing serum obtained from rabbit number 2; 3: wells containing serum obtained from rabbit number 3; red arrows: four constant precipitation lines observed in all cases; green arrows: antigenic community reactions; yellow arrow: additional fifth precipitation line in case of rabbit number 1;)

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In chapter nine entitled „Serological diagnosis of Bordetella bronchiseptica infection in swine and dogs” the aims of the studies were to diagnose Bordetella bronchiseptica infection in pigs and dogs using double immunodiffusion and slow agglutination tests. Also a study was made regarding the sensitivity of these tests in dogs and pigs, meaning that we studied the possibility to use these tests as a routine serological test for Bordetella bronchiseptica infection in dogs and swine.

A total of 534 pig’s serum samples were taken in study. Fifty samples came from 3 months old healthy pigs raised in a farm in Bihor district. For these pigs also bacteriological test were performed to compare the results with serological tests.

A number of 389 samples were positive at the slow agglutination test (72,846%). In case of the 50 pigs from Bihor district three strains of Bordetella bronchiseptica were isolated. Bacteriological positive animals had high but various antibody titre (1:160, 1:320 and 1:1280). Also there were animals with high antibody titre that came out negative at bacteriological test. These results suggest that isolation of Bordetella bronchiseptica from pigs can be correlated with high antibody titre, but high antibody titre does not always mean positive bacteriological test.

Immunodiffusion tests were positive in 10 cases (procentage of positive samples was only 1,875%). All these samples were positive in the slow agglutination test but anti-Bordetella bronchiseptica titres ranged between 1:20 and 1:5120. Also many samples with high antibody titre were negative in the immunodiffusion test. Comparing the results obtained in these two tests we can conclude that there is no correlation between these results and the immunodiffusion test can not be used for diagnose Bordetella bronchiseptica infection in pigs because it lacks sensitivity.

In case of the 63 dogs serum samples and nasal mucus were collected to compare results obtained in bacteriological tests with those obtained in serological tests. Four dogs taken in study suffered of infectious canine tracheobronchitis and in their case the bacteriological exam was positive. They had high anti- Bordetella bronchiseptica antibody titres (1:640-1:1280) and the samples taken from these animals were clearly positive in the immunodiffusion tests too.

These findings showes that infectious canine tracheobronchitis can be diagnosed very easy with both of the serological tests used in our study. With 8 strains of B. bronchiseptica isolated isolation percentage of B. bronchiseptica among the 63 dogs was 12,698%. Four samples were collected from two months old healthy dogs, but in spite of the fact that they did not presented any signs of respiratory distress the anti- B. bronchiseptica antibody titre in these cases were between 1:80 and 1:160. Cultural exam was negative in all four cases. In these cases the antibodies probably has a maternal origin. In case of bacteriological positive but healthy dogs antibody titre always reached titres of
1:80, but relatively high titre (1:80-1:320) does not mean positive culture in healthy dogs.

From the total of 63 serum samples anti- *Bordetella bronchiseptica* antibody titres were higher than 1:10 in 34 cases (53,968%). The immunodiffusion method showed 21 (33,333%) positive samples. There was a corelation between titres equal or above 1:40 and positive reaction in the immunodiffusion test. Samples with titre of 1:40 were inconstant positive but higher titre meaned positive result in the immunodiffusion test.

Comparison of these results shows that titre of 1:40 of anti-*Bordetella bronchiseptica* antibodies can be considered a certenly positive result in dogs, so slow agglutination test can be used as an imunological test for *Bordetella bronchiseptica* infection in this host species. Immunodiffusion test can be also used in serological diagnosis of this infection, but it is less sensitive than the slow agglutination test. Anyway both tests are more sensitive than the bacteriological test. Interpretation of these serological tests must be performed with precaution because they can show false positive results in young animals as well as in vaccinated animals.

The tenth chapter is entitled „Research regarding experimental infection with *Bordetella bronchiseptica* and an acellular anti-*Bordetella bronchiseptica* vaccine efficiency test in rats”. In this chapter we had two obiectives. The firs one was to test the efficiency of an acellular anti-*Bordetella bronchiseptica* vaccine prepared in our laboratory in Wistar rats. The second obiective of this study was to observe the outcome of the intranasal infection with *B. bronchiseptica* in three groups of rats of 3 rats each (a control group of immunocompetent rats, a second group of rats treated with prednisone and the group of the vaccinated rats). We know that immunocompetent rats never develop clinical signs after infection with *Bordetella bronchiseptica* but they will be long term carriers. We suspected that rats treated long time (8 weeks) with high doses of prednisone (5 mg/kg/day) may become immunosupressed and they may develop clinical signs after infection.

Rats from the control group and the group treated with prednisone were infected in the first day of the experiment. The remaining rats were vaccinated subcutaneously in the first and the fifteenth days of the experiment and they were infected only in 15 days after the second vaccine administration. Rats were constantly observed to notice any signs of respiratory distress. The experiment ended after 90 days. The rats were euthanasied, anatomopathologic exam was performed in all rats, blood, nasal mucus and tissue samples (trachea, lung) were collected for serological, bacteriological and histopathological exams.

The vaccine used in this study was prepared exactly as the antigen solutions for the hyperimmunisation of the rabbits.

Clinical, anatomopathological, bacteriological and serological tests showed that that the waccine had a protective efect. Vaccinated rats were *B.
bronchiseptica free and they had high antibody titre as determined with the use of slow agglutination test.

As we expected the rats from the control group were *B. bronchiseptica* carriers, the bacteria could be isolated from their nasal cavity and trachea. In case of the prednisone treated rats *B. bronchiseptica* was isolated from lung tissue in one case and in the case of an another rat *B. bronchiseptica* was isolated in pure culture from the nasal cavity. Even if histopathological exams did not showed exudative lung lesions, our findins suggest that long term treatment with high doses of coricosteroids is a risc factor in case of *B. bronchiseptica* carrier animals.

In chapter 11, entitled „Antibiotic susceptibility tests of the Bordetella strains isolated from animals” a research was made regarding the capacity of 15 antibacterial products to inhibit the growth of the 58 isolated *Bordetella bronchiseptica* and the two isolated *Bordetella parapertussis* strains. These *in vitro* tests were performed using the Kirby-Bauer difusimethric technique.

In case of *Bordetella bronchiseptica* strains ciprofloxacine and enrofloxacine proved to be highly effective with efficiency percent of 98,275% and 93,103%. Beta-lactam antiobotics had no inhibitory efect on *Bordetella bronchiseptica*. 
Image 13, Graphic representation of the sensitivity of the isolated *Bordetella bronchiseptica* strains to different antibiotics;

*Bordetella parapertussis*$_{ov}$ strains had the same sensitivity to antibiotics as the tested *Bordetella bronchiseptica* strains.

In chapter 12, entitled „Research regarding enviromental resistance of the isolated *Bordetella species*” we made a study on the survival capacity of *Bordetella bronchiseptica* and *Bordetella parapertussis*$_{ov}$ in different enviromental conditions. We tested both dry and wet enviromental conditions. In dry conditions both species showew low resistance. Differences appeared in wet conditions (semisolid agar, dechlorinated tape water and Phosphate Buffer Saline, pH 7,2). In dry conditions *Bordetella parapertussis*$_{ov}$ resisted only a
few ours and maximum 21 days in wet, optimal survival conditions. *Bordetella bronchiseptica* can survive 9 months and even multiply in semisolid agar at 4-6 °C. In dechlorinated water this bacteria is able to multiply and survive 300 days. In Phosphate Buffer Saline *Bordetella bronchiseptica* multiplied and resisted 335 days (image 14). These results shows that only *Bordetella bronchiseptica* could have an enviromental reservoir.

![Image 14, Dinamic of *Bordetella bronchiseptica* cell multiplication in tape water and Phosphate Buffer Saline;](image-url)