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Humoral Immune Response Assessment in Sheep Experimentally Infected with *Histophilus somni* and Previously Inoculated with Parainfluenza 3 Virus

[1,2]Laura García Celis, [1,2]Jehieli Girela Álvarez, [1]Beatriz Arellano-Reynoso,
[1]Víctor Tenorio Gutiérrez, [1,2]Francisco Aguilar Romero, [1]Pedro Mejía Sánchez,
[2]Francisco Suárez-Güemes and [1]Efrén Díaz-Aparicio
[1]CENID-Microbiología, INIFAP,
[2]Universidad Nacional Autónoma de México

Abstract: There are no assays of experimental infection of sheep with *Histophilus somni* and their immune response. In order to know the humoral immune response of sheep experimentally infected by respiratory tract with *H. somni*, 19 male, 6 month-old sheep were immuno-suppressed with dexamethasone, 1 day before being inoculated with Parainfluenza 3 virus and during 5 days after viral inoculation. Seven days after viral inoculation, one animal was slaughtered in order to observe lesions caused by the virus. Another 12 animals were inoculated intratracheally with *H. somni* (1×10^9 UFC mL^{-1}), leaving 6 animals as controls that received same pathway sterile PSS. Heart and respiratory rates were taken, as well as rectal temperature, during 14 days after challenge. Serum and nasal exudate samples were collected to determine IgG, IgM and IgA levels by indirect ELISA test, using *H. somni* 40 kDa outer membrane protein as antigen. Two sheep from the inoculated group and one from the control group were humanely euthanized every week during 6 weeks. Samples were collected from lung, tonsils, retropharyngeal and mediastinal lymph nodes; bacteriology studies were carried out in duplicate and DNA was extracted to perform PCR with primers designed for 16S ribosomal region in *H. somni*. Infected animals had temperature and respiratory rate increase, cough and mucopurulent nasal exudate; areas in the apical lung lobule were consolidated and there were adherences as well as. *H. somni* was isolated from only one animal from retropharyngeal node. Nevertheless, from nasal exudate samples, 11 of 12 infected sheep came out positive by PCR while the 6 non-infected controls were negative. ELISA test results for IgG were significantly different between infected and control animals at days 4 and 7 after inoculation, while no differences were found in IgM and IgA. It was concluded that in sheep experimentally infected, *H. somni* caused an IgG isotype humoral immune response. The presence of the bacteria could be detected by PCR in 91.66% of the animals. Even though the strain used in this research was previously passed through sheep, it did not cause lesions or signs that could suggest infection and it could not be recovered by bacteriology in most of the infected sheep.

Key words: *Haemophilus somnus*, *Histophilus somni*, immunoglobulin, sheep, inoculated, humoral immune response

INTRODUCTION

H. somni presence in sheep was first reported in a case of a female with mastitis (Roberts, 1956). Afterwards, Katen *et al.* (1962) isolated it from septicemic lambs. There were other reports in 1977 and bacteria were isolated from a ram with pneumonia, myocarditis and central nervous system lesions (Stephens *et al.*, 1983). In South Africa, isolation was achieved from a lamb with epididymitis and in Canada it has been isolated from vaginal discharge (Stephens *et al.*, 1983; Walker *et al.*, 1988). It has also been reported as causal agent of thrombotic meningoencephalitis in sheep in Australia (Philbey *et al.*, 1991); in 1993, it was isolated from septicemia cases in New Zealand (Kearney and Orr, 1993) and in Mexico there was a report of isolation from a lamb with epididymitis (Palomares *et al.*, 2005).

H. somni isolation from vaginal discharge, as well as prepuce and respiratory tract, is frequent in sheep without clinical manifestations and therefore this microorganism is considered as part of normal microbiota. There is scarce knowledge of infection mechanisms and pathogenicity of *H. somni* in sheep. In a study performed by Ward *et al.* (2006) bacteria that had the same characteristics of

Corresponding Author: Efrén Díaz-Aparicio, CENID Microbiología INIFAP, Carretera México-Toluca, Km 15.5, Palo Alto, Cuajimalpa, D.F. México, C.P. 05110

H. somni were isolated from Rocky Mountain rams, from prepuce, vagina and pneumonic lesions. *H. somni* is an opportunistic pathogen, since it is found associated to factors that favor its colonization with certain bacteria such as *A. seminis*, *Pasteurella multocida* and certain viruses.

Most of the antigens used for serological diagnosis of *H. somni* in sheep have come out as non-specific and in light of the foregoing, a series of studies have begun, testing 40 kDa, Outer Membrane Protein (OMP) as diagnostic antigen or immunogen (Gogolewski *et al*., 1988; Siddaramppa and Inzana, 2004).

The objective of this study was to develop pneumonia, through experimental infection with *H. somni*, as well as evaluate the humoral immune response.

MATERIALS AND METHODS

Bacterial strains: *H. somni* ATCC 2336 strain that was previously inoculated into sheep and recovered from lung was used. To obtain growth it was plated on chocolate agar added with 0.5% yeast extract and 7.5% bovine blood, incubated at 37°C for 48 h in 5 to 10% CO_2 environment.

Antigen: *H. somni* OMP was obtained by the procedure reported by Corbeil *et al.* (1991). Forty kDa OMP was identified and electroelluted at 60 mA for 60 min; a concentration of 41.5 μg mL^{-1} OMP was obtained.

Animal groups and inoculation: Nineteen, 6 month-old Black Belly male sheep were used. Three days before the experiment, they were dewormed with mebendazol (doses 80 mg kg^{-1}). Animals were immunosuppressed with i.m. dexamethasone (3 mg 1.5 mL^{-1}) 1 day before endotracheal challenge with Parainfluenza 3 (PI3) virus at 1×10^{6} Infective $Dose_{50\%}$ in cell culture per milliliter ($CCID_{50\%}$ mL^{-1}), continuing with the dexamethasone treatment during 5 days after challenge. On day seven after viral inoculation, one animal was slaughtered to observe lesions caused by PI3 virus and twelve animals were inoculated intratracheally with *H. somni* (1×10^{9} CFU mL^{-1}), leaving 6 animals as controls that were inoculated with sterile PSS by the same pathway.

Fourteen samplings of blood serum and nasal exudate were performed, with 4 days intervals, beginning 3 days prior to viral challenge. Cardiac and respiratory rates, as well as rectal temperature were taken 3 days prior to PI3 challenge and during all the study.

From day 7 after bacterial challenge on, two animals inoculated with *H. somni* and one control were euthanized per week. Slaughter was performed as established in the Mexican Official Standard (NOM-033-ZOO-1995).

Samples were taken from lungs, tonsils, retropharyngeal and mediastinal lymph nodes, to look for macroscopic lesions.

Bacteriological studies: Tissues and organs that were obtained were plated on chocolate agar in duplicate, as well as the nasal exudate samples; all plates were incubated for 48 h at 37°C in 5 to 10% CO_2 environment, with two independent repetitions.

Serological studies: Indirect ELISA was standardized to titer isotypes, using as antigen 40 kDa OMP and IgG, IgM and IgA anti-immunoglobulins conjugated with peroxidase (BethylL Laboratorios Inc.). Absorbance was measured by a 405 nm filter reader. Positive serum was obtained from one sheep immunized with *H. somni* and the negative from another sheep without apparent respiratory problems and negative by Immunodifution test and also bacteriology.

For IgG, antigen dilution was 1:2000, 1:50 for serum and 1:15,000 for the secondary antibody. IgA isotype measuring was performed from nasal exudates samples, using a 1:250 antigen dilution, 1:20 nasal exudate and 1:1000 conjugate. To determine IgM isotype, optimum antigen dilution was 1:100, 1:50 for serum and 1:1000 conjugate.

PCR: PCR was carried out to determine the presence of *H. somni* DNA in sheep nasal exudates, using 5'-GAAGGCGATTAGTTTAAGAG-3' and 5'-TTCGGGCACCAAGTATTC/A-3' primers (Angen *et al*., 1998). Samples were processed in a thermocycler (Gene Amp PCR System 2400). Thirty five cycles with denaturing at 94°C for 1 min were carried out, with 42°C for 1 min alignment and an extension of 72°C for 1 min (Angen *et al*., 1998). Amplified band detection was evaluated by electrophoresis in 1% agarose gel (Angen *et al*., 1998; Tegtmerier *et al*., 2000).

DNA control was extracted from *H. somni* 2336 ATCC strain. To determine sensitivity of the PCR technique, DNA extractions were carried out from 1×10^{9} to 1×10^{0} CFU mL^{-1} *H. somni* dilutions by phenol-CTAB method (Arellano, 1998). DNA extractions of other bacterial genera related to *H. somni* and that are within the family *Pasteurellaceae*: *Actinobacillus seminis*, *Mannheimia haemolytica*, *Pasteurella multocida* and *Actinobacillus pleuropneumoniae* and *Brucella ovis* were carried out to determine test specificity. DNA was frozen at -70°C until used.

Statistical analysis: A t-test statistical analysis was carried out with a significance value of $p<0.05$, in order to compare ELISA results of infected animals with controls, on days after infection.

RESULTS

Physiological alterations were observed in 46.7% of infected animals. Clinical signs included cough and mucopurulent nasal exudate.

The animal infected with PI3, that was slaughtered before the rest of the animals were inoculated with *H. somni*, had consolidation in an area of the apical pulmonary lobule. In 59% of the cases, sheep that were inoculated with *H. somni* showed small consolidated areas in the cardiac-pulmonary and apical lung lobules as well as emphysema. Two of the 12 infected animals had adherences between pleura and myocardium. Bacteriological studies were carried out with collected tissues and organs of the infected group animals. Bacteria were isolated from retropharyngeal node of only one animal. By serology, IgG isotype had significant difference ($p<0.05$) between infected and control animals, on day 4 and 7 after inoculation. In infected animals when comparing between days, there was a statistically significant difference ($p<0.05$) only on day 7 after inoculation.

For isotype IgA measuring, when comparing different days of infected animals, there were statistically significant differences ($p<0.05$) between days 7, 21 and 36 after infection.

For isotype IgM there was statistical difference ($p<0.05$) on day 11 after challenge with *H. somni*. When comparing between days, statistical difference was found on day 4 after infection ($p\geq0.05$).

Sensitivity detected by PCR performed with bacterial concentration dilutions of *H. somni* of 1×10^{9} to 1×10^{0} CFU mL^{-1}, was 1×10^{1} CFU mL^{-1}, observing a band of approximately 400 pb, that was the expected fragment when using the mentioned primers. When determining PCR specificity, with different bacteria, it was seen that strain *A. seminis*, *P. multocida* and *Actinobacillus pleuropneumoniae* and *B. ovis* did not amplify.

From nasal exudate samples, 11 of 12 infected sheep (91.66%) were positive by PCR and the six non infected controls were negative.

DISCUSSION

Sheep in this study did not develop pneumonic processes. Tegtmeier *et al.* (2000) did a study in bovines by endotracheal inoculation of *H. somni* and *Mycoplasma dispar* observing that the animals were immunosuppressed since leukocyte counts were lowered and pneumonia developed in those animals, while inoculation with only *H. somni* did not cause any lesion and the animals had leukocytosis. Gershwin *et al.* (2005) challenged bovines with Bovine Respiratory Syncytial Virus (BRSV) together with *H. somni* and managed to develop bronchopneumonia.

In this research, the immune system of sheep was depressed by the use of dexamethasone and PI3 and even then, infection by *H. somni* was not established in lungs. Consolidation areas were only observed in the cardiac and apical portions of the lung and *H. somni* isolation was not achieved, so that the lesion could have been caused by PI3. It has been observed that a strain of *H. somni* isolated from bovine pneumonia does not cause the same disease in sheep (Lee *et al.*, 1994) although the electrophoretic profiles of strains isolated in bovines are similar to the strains isolated in clinical cases of sheep (Lee *et al.*, 1994). The strain used in this study was from bovine origin and it was previously inoculated into a sheep that showed small pneumonic lesions at the site of repeated direct inoculations. Perhaps the different origin caused the lack of reproduction of the disease in any of the experimental groups, together with other factors such as environment, handling, feed type, etc. that may predispose the individual to the disease.

In cattle, different strains of *H. somni*, have been isolated; some have been obtained from cases of pneumonia (2336 ATCC), of abortion (649), from encephalon (8025) and prepuce (129Pt); when animals were challenged with strain 8025, pneumonia did not develop and when ELISA tests were performed with susceptible animals' serum, variance between different strains was detected. This indicates that to develop pneumonia in cattle, virulent strains isolated from lung are needed such as strain 2336 ATCC (Groom *et al.*, 1988b).

Ward *et al.* (1995) based on results obtained by ribotype and biotype analysis of different *H. somni* strains isolated from sheep and bovines, came to the hypothesis that strains isolated from bovines would not infect sheep and vice-versa. Therefore, to develop infection in sheep it might be necessary to use a strain isolated from sheep and only the region or anatomical site from where the challenge strain was obtained shall be affected.

By serology, when measuring IgG there was statistically significant difference ($p<0.05$) between both groups, demonstrating that *H. somni* was recognized as a foreign agent, causing a humoral immune response, the same as any other microorganism, but the disease did not develop. In this study, it was observed that 40 kDa OMP was a good antigen for indirect ELISA test, although

previous reports indicate that complete cells give good results also (O'Connor *et al*., 2001; Gershwin *et al*., 2005).

In bovines, IgG2 alotype is considered as the immunoglobulin that gives the highest protection against infection, mainly against pneumonia, since it activates complement and it is an excellent opsonin (Gershwin *et al*., 2005; Siddaramppa and Inzana, 2004).

Results found in this study for IgG and IgM immunoglobulins coincide with reports by Acosta *et al*. (2007) in sheep experimentally infected with *Actinobacillus seminis* with an increase in titers of these isotypes, in the first 2 weeks after inoculation.

Immunoglobulin IgA is an important defense for mucosa and in this research a minimal statistical difference was found ($p<0.05$) between days after inoculation. There is record of calves infected with *H. somni*, where IgA response showed no difference and this is attributed to a crossed reaction with other bacteria of the *Pasteurellaceae* family that are present as opportunistic microorganisms (Gershwin *et al*., 2005).

Isotype IgM manifested a primary humoral response of the animal exposed to *H. somni* as it happens with exposures to any other microorganism.

The presence of *H. somni* in tissues and organs of experimentally infected sheep may be confirmed by PCR. According to Angen *et al*. (1998) when using the same primers as were used in this study, test specificity is adequate since bacteria closely related to *H. somni* such as *A. seminis* and *P. multocida* are not amplified.

Test sensitivity was shown to be good since it amplified *H. somni* DNA that was extracted from 10^1 CFU mL^{-1}.

PCR was more efficacious than the bacteriological studies in demonstrating *H. somni* presence, due to the fact that the test is highly sensitive and needs a smaller amount of bacteria than the bacteriological study in order to indicate infection. Also, it does not require viable or cultivable bacteria to work. This contrasts with the bacteriological studies that in the case of *H. somni* require specific and special growth conditions and for this reason it is considered as a fastidious bacteria.

CONCLUSION

H. somni caused a humoral immune response in infected sheep, developing response against IgG isotype. Presence of *H. somni* in sheep infected experimentally by trachea was demonstrated by PCR in 91.66% of animals; even though the strain used in this research (2336 ATCC) was previously recovered from sheep, lesions were not caused and there were no signs suggesting infection and the bacteria could not be retrieved by bacteriology in most of the challenged sheep.

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