ISSN 1682-8356
ansinet.org/ijps

INTERNATIONAL JOURNAL OF

POULTRY SCIENCE

ANSInet
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

OPEN ACCESS International Journal of Poultry Science

ISSN 1682-8356
DOI: 10.3923/ijps.2018.493.498

Research Article
Using T Cell Lymphokines of Hyperimmunized Chickens with *Salmonella enteritidis* to Protect Neonatal Broiler Chicks Against Infectious Bronchitis Disease

[1]Mushtaq T.B. AL-Zuhariy, [2]D. SaharAbdulhasan, [1]SaharHamdi Abdulmaged and [1]Ihsan M. Shihab

[1]College of Veterinary Medicine, University of Baghdad, Baghdad, Iraq
[2]College of Veterinary Medicine, University of Wasit, Wasit, Iraq

Abstract

Objective: The present study was designed to reduce infectious bronchitis virus (IBV) infections in broiler chickens because commercial vaccines are not able to provide absolute protection due to the absence of cross immunity between strains, as well as recurrent genetic mutations of IBV. Salmonella-immune lymphokines (S-ILK) from hyperimmunized birds with *Salmonella enteritidis* were used to enhance the immune resistance of broiler chicks and to reduce the replication of IBV in infected tissues. **Materials and Methods:** This study was conducted on 250 one-day-old broiler chicks divided into five groups. All groups were treated on the first day as follows: G1: Injected with 0.5 mL S-ILK intraperitoneally and after 30 min challenged with 0.1 mL IBV (variant 2 isolate), G2: Injected with 0.5 mL salmonella-nonimmune lymphokines (S-NILK) intraperitoneally and after 30 min challenged with 0.1 mL IBV (variant 2 isolate), G3: Only injected with 0.5 mL S -ILK intraperitoneally without challenge with IBV, G4: Only challenged with 0.1 mL IBV (variant 2 isolate) and G5: Not injected and not challenged, considered a negative control group. **Results:** The results showed a significant increase ($p<0.05$) in the antibody titer of all treated groups. G1 showed a moderately significant increase in the antibody titer and the number of RNA copies of IBV, as well as the lowest morbidity and mortality rates during the trial period. G4 , followed by G2, recorded a highly significant increase ($p<0.05$) in the antibody titer and number of RNA copies of IBV as well as high morbidity and mortality rates compared to G3 and G5, which did not record any result due to non-exposure to IBV. **Conclusion:** It is concluded that giving S-ILK at an early age enhances maternal immunity against IBV infection and prevents viral replication in the tracheal tissue after challenge with IBV (variant 2 isolate). We thus can save time, effort and resources in vaccination operations that do not provide absolute protection against IBV infections.

Key words: *Salmonella enteritidis*, immune lymphokines, infectious bronchitis, ELISA, viral load

Received: July 16, 2018 Accepted: September 05, 2018 Published: September 15, 2018

Citation: Mushtaq T.B. AL-Zuhariy, D. SaharAbdulhasan, SaharHamdi Abdulmaged and Ihsan M. Shihab, 2018. Using T cell lymphokines of hyperimmunized chickens with *Salmonella enteritidis* to protect neonatal broiler chicks against infectious bronchitis disease. Int. J. Poult. Sci., 17: 493-498.

Corresponding Author: Mushtaq T.B. AL-Zuhariy, College of Veterinary Medicine, University of Baghdad

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Infectious bronchitis is a highly acute viral contagious disease in poultry caused by the corona virus belonging to the family of Coronaviridae. It is characterized by depression, tracheal rales, sneezing, coughing, wet frothy eyes with conjunctivitis and very high morbidity and mortality rates in a very short time, accompanied by secondary infections, it causes major economic losses in the global poultry industry[1]. The incidence of infectious bronchitis virus (IBV) in many countries around the world changes over time due to the virus having a tendency toward genetic mutations, resulting in variant strains possibly having genome changes[2]. Mutations are the natural mechanisms of IBV, IBV attempts to evade the host immune system defenses, which results in variant strains not distinguishable by new neutral epitopes because antibodies are produced from repeated vaccines. The lack of cross immunity between some IBV serotypes against variant strains is unexpected[3]. Frequent IBV infections are difficult to control, although many commercial vaccines in different forms (attenuated and killed) have been used to reduce the incidence of IBV. These strategies have not been able to provide absolute protection against IBV infection because more variant strains have been caused by frequent genetic mutations responsible for recent outbreaks[4]. Many studies have indicated a weak immune response produced from vaccines against IBV[5,6]. The development of the immune response is associated with complex interactions between hematopoietic, inflammatory and lymphoid cells[6]. Cytokines are small molecules composed of proteins and glycoproteins; they are biologically active proteins that interact with many different resistance mechanisms and as major carriers in inflammatory reactions[7]. Because of the continued exposure to IBV and multiple vaccination programs, the use of antiviral and antibiotics has not succeeded in effectively reducing IBV. The use of lymphokines to enhance immune resistance gives a greater protective solution in preventing outbreaks of serious IBV infections[8,9]. The present study was conducted to show the effective role of salmonella-immune lymphokines (S-ILK) in reducing the negative effects of infectious bronchitis in broiler chickens[10].

MATERIALS AND METHODS

Preparation of lymphokines: An unpublished *S. enteritidis* isolate was obtained from the Department of Poultry Diseases, College of Veterinary Medicine, University of Baghdad. The isolate was grown in nutrient broth and peptone water. The colonies and white sediments appeared after 24 h, some colonies were taken to grow on selective media for salmonella, such as MacConkey and SS agar and showed black colonies. The isolate was identified biochemically using the API20 test. Finally, a bacterial solution with a concentration of 1×10^8 colony forming units mL^{-1} was prepared spectrophotometrically in a standard curve at a wavelength of 625 nm.

In the second step, 100 broiler chicks at 1 day of age were divided into two groups. The first group was treated orally with three doses of bacterial solution (1×10^8 colony forming units) (7, 14 and 21), group 2 was treated with PBS in the separate breeding unit and was considered a control group. The spleen was collected from immunized birds after slaughter in both groups at 30 days. The spleen was placed in petri dishes containing PBS and then cut and crushed. A centrifuge was used to separate the mixture. T-Lymphocyte cells were implanted in tissue culture, T cells were stimulated using con-A to secrete lymphokines and S-ILK was collected from T cells of spleen from the first group. Salmonella-nonimmune lymphokines (S-NILK) were collected from the T cells of the spleen from the second group. The preparation and separation process was performed according to Kogut *et al.*[11].

Viral inoculum: The unpublished isolate of IBV (variant 2) (allantoic fluid) from the Department of Poultry Diseases, College of Veterinary Medicine, University of Baghdad, was grow on chicken embryos for 9-11 days and allantoic fluid was collected from infected eggs, the lethal dose of the virus (ELD_{50} 10^5) was determined according to a previously published method[12].

Experimental design: The study was conducted on 250 1-day-old broiler chicks divided into five groups. Whole groups were treated on the first day as follows: G1: Injected with 0.5 mL S-ILK intraperitoneally and after 30 min challenged with 0.1 mL IBV (variant 2 isolate) (100 ELD_{50} 10^3), G2: Injected with 0.5 mL S-NILK intraperitoneally and after 30 min challenged with 0.1 mL IBV (variant 2 isolate), G3: Only injected with 0.5 mL S-ILK intraperitoneally without challenge with IBV, G4: Only challenged with 0.1 mL IBV (variant 2 isolate) and G5: Not injected and not challenged, considered a negative control group. All chicks were observed daily and their clinical symptoms and mortality were recorded.

Sampling collection: Using non-anticoagulant tubes, blood samples were collected from the jugular vein of five chicks

from each group on days 7, 14, 21 and 28. The serum was separated from the blood samples by centrifugation at 1000 rpm for 15 min. Serum was stored in the freezer (-20 m) until laboratory use. Samples of tracheal tissue were collected after the postmortem of infected birds; five samples of each group (days 7, 14, 21 and 28) were stored in a deep freezer (-80 m) for laboratory use.

Clinical symptoms and mortality: Clinical symptoms were recorded during the experimental period after challenge with the IBV (variant 2) strain. Respiratory symptoms were cough, sneezing, rales and wheezing. The other symptoms were dehydration, lethargy, emaciation and prostration. Pathological symptoms were catarrhal exudate in the sinuses and nasal cavity, congestion of blood vessels in the trachea, material bifurcation of the trachea and primary bronchial, paleness and hypertrophy of the kidneys and urate deposit.

Real-time RT-PCR: According to a previously published method of Sun *et al.*[13], the number of IBV RNA copies in the tracheal tissue of infected birds was calculated. RNA was extracted from the tracheal tissue of infected birds by using TRIZOL Reagent (Invitrogen, Carlsbad, CA, USA). Specific primers and TaqMan probes were used against IBV RNA for virus detection[14]. Real-time RT-PCR was performed depending on the LightCycler® 480 real-time PCR system (Roche Diagnostics Deutschland GmBH, Mannheim, Germany) using a one-step PrimeScript RT-PCR kit[13].

ELISA test: The ELISA test was used to identify IBV antibodies in the bird serum. The protocol was used according to the ProFlock® IBV ELISA kit[15].

Statistical analysis: Statistical Analysis System (SAS) was used to analyze the results of the current study and to evaluate the effects of the different factors in the study parameters[16]. The LSD test was used to determine the least significant difference between the results; significance was evaluated at the level of $p<0.05$.

RESULTS

Immune resistance against IBV: Maternal immunity was determined on the first day by examining 10 serum samples by ELISA before the chicks were divided into groups. The results showed good maternal immunity (10325±365). Table 1 shows the results of ELISA in determining the antibody titer against IBV. On day 7, all treated groups showed a significant decrease ($p<0.05$) in the antibody titer when compared with those in G3 and G5, which showed average maternal immunity. A significant increase ($p<0.05$) was observed on days 14 and 21 in G1, followed by G2, while G3 and G5 showed a significant decrease ($p<0.05$) in maternal immunity reaching zero compared to that in G4, which did not score any result. On the 28th day, G4 recorded the highest significant increase ($p<0.05$) with antibodies, followed by G2, while G1 showed a moderate increase ($p<0.05$) compared to G3 and G5.

Viral load: Table 2 shows the results of RT-PCR after IBV (variant 2), which included a significant increase in the RNA copies of IBV (variant 2) in the first day. On day 28, G4 showed the highest significant increase ($p<0.05$) in the number of RNA copies of IBV, followed by G2, while G1 recorded the lowest increase compared to G4 and G2. G3 and G5 did not report any lesions due to non-exposure to IBV.

Morbidity and mortality: Table 3 shows the clinical signs recorded during the experimental period after challenge with IBV (variant 2) on the first day. Respiratory symptoms included sneezing, coughing, rales and wheezing. Other signs included dehydration, depression, emaciation and prostration. The highest morbidity and mortality rates were recorded in G4, reaching approximately 100% of the total 50 chicks, followed by G2. In contrast, G1, which was treated with S-ILK, had the lowest morbidity of approximately 25% and no mortality. G3 and G5 did not report any lesions due to non-exposure to IBV (variant 2).

Table 1: The antibody titer against IBV (Mean±SE) after challenge with IBV (variant 2 100 ELD_{50} 10^3) strain in broiler chickens as determined by ELISA

Periods	7 days	14 days	21 days	28 days
IBV antibody titer mean±Standard error (groups)				
G1	6336.5±211.3^B	3299.2±158.4^A	5659.5±244.7^A	6324.7±562^C
G2	4877.2±196.5^C	2221.8±177.5^B	6568.9±210^B	16566.8±654^B
G3	7765.1±227.5^A	1210±122.3^C	0±0^C	0±0^D
G4	4766.5±187.4^C	0±0^D	0±0^C	23782.8±789.9^A
G5	7897.3±266.7^A	1311±132.4^C	0±0^C	0±0^D
LSD	622.28	326.53	355.36	3803.58

No. of samples: 5 from each group, Capital letters indicate significant differences ($p<0.05$) between the treatment means, LSD: Least significant difference

Table 2: The results of RT-PCR for IBV RNA copies (Mean±SE) after challenge with IBV (variant 2 100 ELD_{50} 10^3) strain in broiler chicken tracheal tissue

Periods	7 days	14 days	21 days	28 days
Viral load Mean±Standard error (groups)				
G1	564.2±18.16^C	922.4±48^C	1321.6±132.6^C	1907.2±121^C
G2	1343.8±48.5^B	2132.2±98.9^B	4353.6±232.6^B	5464.6±232.4^B
G3	0±0^D	0±0^D	0±0^D	0±0^D
G4	2314.4±66.5^A	3911±123.8^A	6575.2±657.8^A	8546.7±392.5^A
G5	0±0^D	0±0^D	0±0^D	0±0^D
LSD	393.82	657.27	1324.34	2543.43

No. of samples: 5 from each group, Capital letters indicate significant differences ($p<0.05$) between the treatment means, LSD: Least significant difference

Table 3: The percentage of clinical symptoms in the treated groups during the experiment after challenge with IBV (variant 2 100 ELD_{50} 10^3) strain in broiler chickens

Signs/groups	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)
Respiratory	5/50 (10)^C	45/50 (85)^B	0/50 (0)^D	50/50 (100)^A	0/50 (0)^D
Others	12/50 (24)^C	25/50 (50)^B	0/50 (0)^D	50/50 (100)^A	0/50 (0)^D
Mortality	0/50 (0)^B	50/50 (100)^A	0/50 (0)^B	50/50 (100)^A	0/50 (0)^B

Respiratory symptoms include sneezing, coughing, rales and wheezing, Other symptoms include dehydration, depression, emaciation and prostration

Table 4: The percentage of pathogenic lesions in broiler chickens treated after challenge with IBV (variant 2 100 ELD_{50} 10^3) strain

Lesions/groups	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)
Respiratory	3/50 (6)^C	25/50 (50)^B	0/50 (0)^C	50/50 (100)^A	0/50 (0)^C
Renal	1/50 (2)^C	20/50 (40)^B	0/50 (0)^C	30/50 (60)^A	0/50 (0)^C
Others	3/50 (6)^C	10/50 (20)^B	0/50 (0)^C	50/50 (100)^A	0/50 (0)^C

Respiratory lesions include catarrhal exudate in the sinuses and nasal cavity, congestion of blood vessels in the trachea and material bifurcation of trachea and primary bronchial, Renal lesions include paleness and hypertrophy of the kidneys and urate deposit, Others include petechial hemorrhage, splenomegaly and moderate enlargement of bursa of Fabricius

Table 4 shows the pathological changes recorded during the experimental period after challenge with IBV (variant 2) on the first day. Respiratory lesions included catarrhal exudate in the sinuses and nasal cavity, congestion of blood vessels in the trachea and material bifurcation of the trachea and primary bronchial, paleness and hypertrophy of the kidneys and urate deposit. Other symptoms include petechial hemorrhage, splenomegaly and moderate enlargement of the bursa of Fabricius. The highest percentage of lesions was recorded in G4, reaching approximately 100% of the total 50 chicks, followed by G2. G1 group with S-ILK had the lowest percentage of pathological changes and did not exceed 6%, in contrast to G4 and G2. G3 and G5 did not report any lesions due to non-exposure to IBV (variant 2).

DISCUSSION

On the first day, the ELISA results provided 10 serum samples with good maternal immunity before dividing the chicks into groups (10325±365). This finding agreed with Mockett and Cook[17], who indicated the cumulative effect of antibodies due to the repeated vaccinations of breeders, as a large amount of antibodies are transferred by the eggs to the chicks. Da Silva Martins *et al.*[18] demonstrated that the high levels of antibodies transferred to the chicks began to gradually decrease to half the original levels after 5 days. Promkuntod *et al.*[19] demonstrated that maternal immunity decreased by two-fold every 4-5 days. At 7 days, they showed a decrease in IBV antibodies compared to G3 and G5, which showed normal levels of maternal immunity, due to neutralization between virus and antibodies. These results agreed with the results of Raj and Jones[20], who referred to the serological neutralization that occurs as a result of interaction between the challenge virus and maternal antibodies on the first day. This significantly reduces maternal immunity, especially if the virus is highly pathogenic. The findings of the current study showed the biological functions of S-ILK in enhancing immune resistance against IBV (Variant 2). The results agree with Hilton *et al.*[21,] who demonstrated that lymphokines are small protein and glycoprotein molecules that are soluble and have small molecular weights. Lymphokines are produced widely by different defensive cells and play roles as hematopoietic and inflammatory agents that help support the inflammatory process through the proliferation, differentiation, activation and attraction of inflammatory cells toward the infective antigen. Arai *et al.*[22] also noted that lymphokines regulate the host immune response against infection, regulating signaling between defense cells; they are produced from T, B, macrophage and dendritic cells in response to the recognition of exotic antigens or their products. The lymphokines were identified as messenger molecules that controlled and regulated the immune cells of both natural and acquired immune responses[23]. These results agreed with Seo and Collisson[24] who noted the role of lymphokines in preventing IBV by increasing the production of lymphocytes and aggregation

in the infected tissues, determining the nature of the humoral immune response that activates and prevails after IBV infections and preventing virus replication in the infected tissue. Asif *et al.*[25] confirmed that S-ILK have an immunological, antiviral and antibacterial role. Collission *et al.*[26] also illustrated the essential role of lymphokines in preventing the replication of IBV in infected tissues, including IFN-γ produced by CD4 T and natural killer defense cells, which plays a role in the elimination of IBV and stimulates the CD8 T and cytotoxic lymphocyte T cells to kill and clean infected tissue from the virus[22].

CONCLUSION

The current study demonstrated the absolute protection provided by S-ILK against IBV by enhancing immune resistance and preventing the replication of IBV in the tracheal tissue after a challenge with field IBV (variant 2) strain at an early age, without the use of attenuated or killed vaccines.

SIGNIFICANCE STATEMENT

The current study revealed the effective role of S-ILK from hyperimmunized birds with *S. enteritidis* in increasing the immune resistance of broiler chicks challenged with field (IBV) (variant 2) strain and preventing viral replication in tracheal tissue. This study helped researchers identify the nonspecific immunity generated by subclinical infections of salmonella in neonatal hatching chicks by reducing acute respiratory infections, which many researchers were not able to detect. Our new theory uses S-ILK to reduce respiratory infections, such as IBV. Because of the time, effort, cost and lack of gross immunity among all vaccinal IBV isolates, these previous strategies have been unable to prevent the replication and outbreak of IBV.

REFERENCES

1. Al-Beltagi, S.E., H.A. Torkey and M.E. Seddeek, 2014. Antigenic variations of infectious bronchitis virus from broiler flocks in Al Behera governorate. Alexandria J. Vet. Sci., 40: 44-51.
2. Susan, S.E.M., M.M. El-Hady and Y.A. Soliman, 2010. Isolation and characterization of nephropathogenic strain of infectious bronchitis virus in EGYPT. J. Am. Sci., 6: 669-675.
3. Antarasena, C., B. Nuansrichay, S. Thongmee and M. Intarasarn, 2008. S1 Glycoprotein gene analysis of infectious bronchitis virus strains isolated in Southern Thailand. Proceedings of the 15th Congress of FAVA-OIE Joint Symposium on Emerging Diseases, October 27-30, 2008, Bangkok, Thailand, pp: 147-150.
4. Okino, C.H., A.C. Alessi, M.D.F.S. Montassier, A.J.D.M. Rosa, X. Wang and H.J. Montassier, 2013. Humoral and cell-mediated immune responses to different doses of attenuated vaccine against avian infectious bronchitis virus. Viral Immunol., 26: 259-267.
5. Nascimento, I.P. and L.C.C. Leite, 2012. Recombinant vaccines and the development of new vaccine strategies. Braz. J. Med. Biol. Res., 45: 1102-1111.
6. Pei, J., M.J. Sekellick, P.I. Marcus, I.S. Choi and E.W. Collisson, 2001. Chicken interferon type I inhibits infectious bronchitis virus replication and associated respiratory illness. J. Interferon Cytokine Res., 21: 1071-1077.
7. Kogut, M.H., R.B. Moyers and J.R. DeLoach, 1996. Las citocinas y sus interrelaciones funcionales: Potenciacion de la respuesta inflamatoriaen el control de infeccionessiste micas por Salmonella en las aves. Memorias del Curso de Actualizacion Avancesen Inmunologia Aviar, Mexico, DF. Asociacion Nacional de Especialistasen Ciencias Avicolas de Mexico, AC, Mexico, DF., March 15, 1996, pp: 8-12.
8. Stern, A.M. and H. Markel, 2005. The history of vaccines and immunization: Familiar patterns, new challenges. Health Affairs, 24: 611-621.
9. Liu, Q., L.C. Miller, F. Blecha and Y. Sang, 2017. Reduction of infection by inhibiting mTOR pathway is associated with reversed repression of type I interferon by porcine reproductive and respiratory syndrome virus. J. Gen. Virol., 98: 1316-1328.
10. Tellez, G.I., M.H. Kogut and B.M. Hargis, 1993. Immunoprophylaxis of *Salmonella enteritidis* infection by lymphokines in Leghorn chicks. Avian Dis., 34: 1062-1070.
11. Kogut, M.H. and T.S. Slajchert, 1992. T lymphocytes induce protection in chickens against *Eimeria tenella* by production of lymphokines. Immunol. Infect. Dis., 2: 69-80.
12. Reed, L.J. and H. Muench, 1938. A simple method of estimating fifty per cent endpoints. Am. J. Epidemiol., 27: 493-497.
13. Sun, J., Z. Han, Y. Shao, Z. Cao, X. Kong and S. Liu, 2014. Comparative proteome analysis of tracheal tissues in response to infectious bronchitis coronavirus, Newcastle disease virus and avian influenza virus H9 subtype virus infection. Proteomics, 14: 1403-1423.
14. Wise, M.G., D.L. Suarez, B.S. Seal, J.C. Pedersen and D.A. Senne *et al.*, 2004. Development of a real-time reverse-transcription PCR for detection of Newcastle disease virus RNA in clinical samples. J. Clin. Microbiol., 42: 329-338.
15. Synbiotic® Corporation, 2005. Newcastle disease virus antibody test kit. Proflock R. Plus, Item No. 96-95 33, Frontera, San Diego, CA., USA.
16. SAS., 2012. SAS User's Guide: Statistics. Version 9.1, SAS Institute Inc., Cary, NC., USA.
17. Mockett, A.P.A. and J.K.A. Cook, 1986. The detection of specific IgM to infectious bronchitis virus in chicken serum using an ELISA. Avian Pathol., 15: 437-446.

18. Da Silva Martins, N.R., A.P.A. Mockett, A.D.T. Barrett and J.K.A. Cook, 1991. IgM responses in chicken serum to live and inactivated infectious bronchitis virus vaccines. Avian Dis., 35: 470-475.
19. Promkuntod, N., R.E.W. van Eijndhoven, G. de Vrieze, A. Grone and M.H. Verheije, 2014. Mapping of the receptor-binding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus. Virology, 448: 26-32.
20. Raj, G.D. and R.C. Jones, 1996. Local antibody production in the oviduct and gut of hens infected with a variant strain of infectious bronchitis virus. Vet. Immunol. Immunopathol., 53: 147-161.
21. Hilton, L.S., A.G.D. Bean and J.W. Lowenthal, 2002. The emerging role of avian cytokines as immunotherapeutics and vaccine adjuvants. Vet. Immunol. Immunopathol., 85: 119-128.
22. Arai, K.L., F. Lee, A. Miyajima, S. Miyatake, N. Arai and T. Yokota, 1990. Cytokines: Coordinators of immune and inflammatory responses. Annu. Rev. Biochem., 59: 783-836.
23. Kaiser, P., 2010. Advances in avian immunology-prospects for disease control: A review. Avian Pathol., 39: 309-324.
24. Seo, S.H. and E.W. Collisson, 1997. Specific cytotoxic T lymphocytes are involved in *in vivo* clearance of infectious bronchitis virus. J. Virol., 71: 5173-5177.
25. Asif, M., K.A. Jenkins, L.S. Hilton, W.G. Kimpton, A.G. Bean and J.W. Lowenthal, 2004. Cytokines as adjuvants for avian vaccines. Immunol. Cell Biol., 82: 638-643.
26. Collisson, E.W., J. Pei, J. Dzielawa and S.H. Seo, 2000. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev. Comp. Immunol., 24: 187-200.