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Volume 1 | Issue 2 | December 2019 | Page 1-45
Veterinary Science Research

Contents

Article

Review

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Veterinary Science Research
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ARTICLE

Determination of the Administration Routes, Doses and Appropriate Age to Vaccinate With Ornitin Triple Vaccine For Cross-breed Colored Broilers in Vietnam

Nguyen Manh Ho Bui Ngoc Xuan Ha Le Thi Kim Tuyen Le Thi Anh Thu Le Thanh Hien Quach Tuyet Anh*

Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh City, Vietnam

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ABSTRACT

The study was to determine the appropriate dose and administration route of Ornitin Triple vaccine in cross-breed coloured broilers in Vietnam by evaluating the antibody titer against *Ornithobacterium rhinotracheale* (ORT) and local reactions at injection sites on chickens after vaccination. The study was divided into 2 trials. Both trials were designed with 3 vaccine dose groups: 0.0ml (control group), 0.25ml and 0.5ml and 2 different administration routes: subcutaneous at neck (SC) and intramuscular at breast (IM) injection. The result showed that, no statistically significant difference was found between antibody titer of two administration routes as well as 2 vaccine doses until 13-week-old. Local reactions at the injection sites of IM route was less severe than SC at neck and in higher dose would produce a more severe swelling reaction. Daily weight gain was found to have a slight decrease in the vaccinated groups within 2 weeks after vaccination, however, no statistically significant difference was found in later stage ($P > 0.05$). In conclusion, Ornitin Triple can be used to vaccinate by IM with the dose of 0.25ml for coloured broilers at early age (3-week-old), or 0.5ml for older birds and should be careful for some reactions at the injection sites.

1. Introduction

Respiratory diseases are the most common among the considerably difficult problems and account for a high proportion in poultry diseases. There are many different causes for respiratory diseases including microorganism factors and farms' management [8]. Among those, bacteria *Ornithobacterium rhinotracheale* (ORT) plays an important part in causing complex respiratory syndromes with high mortality [15]. Diversity of ORT with 18 serotypes (type A to type R), serotype A was the most common with 94% [4,7]. Beside that, a survey on random samples, Numee et al found that 3 common serotypes were A (35.5%), B (19.4%), and C (12.9%) [11]. Unlike other repiratory causes, ORT was discovered rather late; it was not known until 1981; the first case of ORT infection was found in turkeys in Germany [3]. Mortality rate of ORT was from 1% to 15% in chickens aged 2-8 weeks old; however, in cases where ORT was followed by a sencondary infection, mortality rate could reach up to 50% [5]. Common clinical signs caused by ORT include coughing, nasal discharge, labored breathing, decreased

**Corresponding Author:*
Quach Tuyet Anh,
Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh City, Vietnam;
Email: lethianhthu9694@gmail.com

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feed and water intake and could lead to death [9]. In breeders, the disease could cause the flock to decrease its egg production as well as produce weak offsprings [5]. As ORT is a slow grower and require a really special growing environment, other bacteria (such as *E.coli*), which have the ability to grow fast and easily, would be dominant in the infected organs [12]. Moreover, ORT is usually a secondary infection to other diseases such as Infections bursal disease (IBD), Newcastle (ND) and Infectious Bronchitis (IB). These are all factors contributing to the late or misdiagnosis and hinder prevention of ORT infection and therefore would leave a negative impact on the economy. In 1998, according to *John R. Glisson*, treatment with antibiotics was more efficient than using vaccine [8]. However, one year later, Van Empel and Hafez (1999) found that treatment of ORT with antibiotics would be extremely difficult due to the fact that most ORT strains had already developed antibiotic resistance [16]. In 2001, Devries et al found that ORT bacteria strains were already resistant to 80-100% of commonly used antibiotics in treating poultry, namely ampicillin, ceftiofur, tylosin, spiramycin, lincomycin, tilmicosin, flumequine, enrofloxacin and doxycycline [6]. As a result, deeper research into ways to prevent the disease might be a more effective solution against ORT. Van Empel et al. (1999) carried on a study on the ORT vaccine with an oil adjuvant capable of reducing clinical signs of the disease. From that, this study carried on to evaluate the serum HGA and the reaction at the injection site of the chicken after vaccination [16]. In 2002, Cauwerts et al conducted an experiment on vaccine against ORT in Belgium and brought about good results such as a decrease in mortality rate and an increase in poultry production performance [2]. In 2005, Schuijffel et al proved the ability to generate cross protection against different serotypes of ORT [14]. However, bacterial vaccines are often seen causing strong reactions in poultry as well as the level of immune response largely depends on the reaction at the injection sites and vaccine antigen [13]. As a result, this research was the first in Vietnam to determine the appropriate doses, administration routes and age in order to simultaneouly minimize the side effects and be able to produce a high and prolonged antibody titer to protect local cross-breed broilers from ORT.

2. Materials and Methods

2.1 Materials

Ornitin Triple (Phibro Animal Health, USA) is an oil emulsion vaccine. In the dose of 0.5ml, the antigen amount consists of 10^9 CFU for each serotype A, B and C. Vaccine is recommended to use in prevention of respiratory diseases caused by ORT. Information of vaccine bottle was used in this study: the batch number: 24461034 and expiry date: 23-05-2020.

Cross-breed colored broilers chickens in Vietnam or namedly backyard chickens or native chickens are raised for 14-16 weeks, with live body weigh at slaughter time in average for 2 – 2.5 kg/bird.

2.2 Methods

2.2.1 Experimental Design

The research consisted of 2 trials:

Trial I: 108 3-week-old local cross-bred broilers

Trial II: 120 5-week-old local cross-bred broilers

All chickens in both trials were tagged individually on the legs. They were initially weighed, measured for ORT antibody before vaccinated and randomly allocated into six groups according to 2 factors shown in Table 1.

(1) 0.0ml represented control group, injected with 0.5ml saline solution (NaCl 0.9%).

(2) 0.25ml represented test group injected with 0.25ml Ornitin Triple vaccine.

(3) 0.5ml represented test group injected with 0.5ml

Table 1. Experimental design

	Trial I: ORT vaccination at 21 days old							**Trial II: ORT vaccination at 35 days old**					
	Subcutaneous (SC)			**Intramuscularly (IM)**				**Subcutaneous (SC)**			**Intramuscularly (IM)**		
Dose (ml) Group	0.0	0.25	0.5	0.0	0.25	0.5		0.0	0.25	0.5	0.0	0.25	0.5
Bird/ cage	9	9	9	9	9	9		10	10	10	10	10	10
No. cage	2	2	2	2	2	2		2	2	2	2	2	2
Total bird	18	18	18	18	18	18		20	20	20	20	20	20
No. blood sample	10	10	10	10	10	10		10	10	10	10	10	10
Average BW (g/bird)	312.7	312.1	312.3	312.5	312.1	312.5		760.1	759.6	763.1	761.5	760.4	759.2

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Ornitin Triple vaccine.

(4) SC represented subcutaneous injection at neck.

(5) IM represented intramuscular injection at breast.

Experimented chickens were weighed and put into groups in each trial. Blood samples were taken from 10 chickens in each group.

2.2.2 Effects of Administration Routes, Doses and Age to ORT Antibody Titer

In each experiment, a total of 18-20 chickens were numbered and 10 were randomly selected for serum sample. Those 10 chickens were recorded of their tag number for later reference. Until they reached the age of 13 weeks old, in both experiments, 5 serum samples were collected in each cage. There were 30 serum samples in each trial collected each collecting time. A total of 600 serum samples from both trials were taken during the study.

ORT antibody titer in chickens' serum samples was tested using ELISA technique by IDEXX commercial kit, IDEXX ORT Ab Test, USA, product code 99-43600. According to the recommendation of the manufacturer, grouping of ORT antibody titer was shown in Table 2 and samples with titer ≤ 844 would be considered as negative, and samples with titer ≥ 844 would be considered as positive (IDEXX, USA).

2.2.3 Evaluation of the Safety of Ornitin Triple vaccine

The safety of the vaccine was evaluated by chickens' general body reactions and local reactions at the injection sites after vaccination. Chickens' general body reactions of each group were assessed by body temperature and feed intake. Body temperature of chickens was measured using Amrus thermometer from 4PM to 6PM on day 1, 2, 4, 5, 6, 8, 10, 14 after vaccination. Each group's feed intake was also recorded for 14 continuous days after vaccination. Chickens' local reactions at the injection sites were observed and scored: no swelling = 0 point, swelling less than 1cm = 1 point, swelling from 1-2 cm = 2 points, and swelling more than 2 cm = 3 points.

2.2.4 Evaluation of Vaccine's Effects to Chickens' Performance

Chickens were weighed and recorded individually; their daily average weight gain (DAWG) was calculated using the following formula:

$$DAWG = \frac{Weight\ (after) - Weight\ (before)}{Age\ (after) - Age\ (before)}$$

2.3 Experimental Data Analysis

Data was collected and managed by MS Excel 2010 (Microsoft, USA). Analyzed by T-student test for 2 group of trials, and by ANOVA variance analysis method of software R version 6.3.1.

3. Results and Discussions

3.1 Evaluation of the Level of ORT Antibody Titer before and after Vaccination

Before vaccination, ORT antibody titer of chickens from all groups were recorded as negative (titer ≤ 844). According to IDEXX, chickens that were not vaccinated against ORT and not infected with ORT by field strains would be considered as negative against ORT and therefore, be classified into group 0 (Figure 1, Figure 2) (Table 2).

The results of chickens' ORT antibody titer 3 weeks after vaccination were displayed in Figure 1 and 2. There was a significant difference between vaccinated groups and the control group in terms of ORT antibody titer. In trial I, 3 weeks after vaccination (chickens reached the age of 6 weeks), their ORT antibody titers would be classified into group 8 and above, with the average titer belonging to group 13 or 14, equivalent to 19000 – 20000 titer unit. In trial II, 3 weeks after vaccination (chickens reached the age of 8 weeks), the lowest ORT antibody titer was recorded at group 15 range with the average titer falling into group 16 or 17. In figure 1, titers ranged from group 8 to 16 while in figure 2, titers ranged from group 15 to 18. This result showed that older chickens can produce higher

Table 2. Grouping of ORT antibody titer (xChekPlus software, IDEXX, USA)

Grouping	**0**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Antibody titer	0-844	845 -999	1000 -1999	2000 -2999	3000 -3999	4000 -4999	5000 -5999	6000 -7999	8000 -9999	10000 -11999
Grouping	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	
Antibody titer	12000 -13999	14000 -15999	16000 -17999	18000 -19999	20000 -21999	22000 -23999	24000 -27999	28000 -31999	≥32000	

and more uniform ORT antibody titers 3 weeks after vaccination.

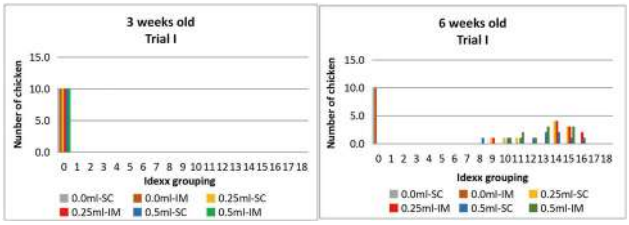

Figure 1. Grouping of ORT antibody titer of chickens at 3 weeks old (before vaccination) and 3 weeks after vaccination in Trial I

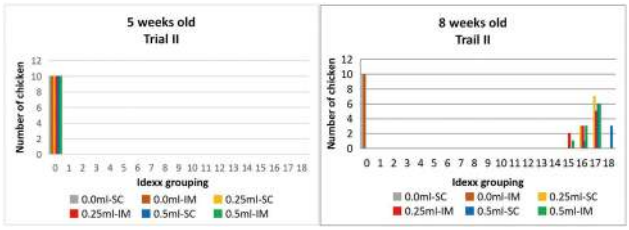

Figure 2. Grouping of ORT antibody titer of chickens at 5 weeks old (before vaccination) and 3 weeks after vaccination in Trial II

3.2 ORT Antibody Titer Post Vaccination

Each group's ORT antibody titers at different ages from trial I and II were recorded in Table 3 and 4, respectively. Before vaccination, ORT antibody titers of all groups were similarly low and not significanly difference ($P > 0.05$). After vaccination, both trials' vaccinated groups experienced an increase in antibody titer compared to the control groups ($P < 0.001$). In addition, 2 weeks after vaccination, both trial I and trial II had ORT antibody titers surged significantly, while in control groups, all were in negative group (Table 3, Table 4).

3.2.1 Effects of Administration Routes to ORT Antibody Titer

According to Bermudez (2008), vaccines needed to be administered at the correct sites in order to achieve the best immune response for poultry [1]. Different type of vaccine would require a different optimal injection site. The effects of administration routes were evaluated based on assessing groups with the same administration dose of each trial. In trial I, with the vaccine dose of 0.25 ml, ORT antibody titer of SC route was not different from that of IM route; similarly, there was no statistical significance between 2 administration routes with the vaccine dose of 0.5 ml ($P > 0.05$) (table 3). The same situation applied to trial II when assessing 2 administration routes with no difference found ($P > 0.05$) (table 4). It can be concluded that, SC or IM injection does not affect the ORT antibody titer of coloured broilers, as there was no significant difference found between the 2 injection routes. Although, there was no statistically difference between SC and IM, it should be carefully to use IM at breast route for broilers to be slaughtered less than 6 weeks after vaccination because IM injection will damage inside the muscle and leave consequences on the carcass quality.

3.2.2 Effects of Vaccine Doses to ORT Antibody Titer

The effect of injection dose on the antibody titer was as-

Table 3. Effects of administration routes and doses to ORT antibody titer after vaccinating with Ornitin Triple vaccine – Trial I

		Subcutaneous (SC)			Intramuscularly (IM)			F_{-value} P_{-value}
		0.0 ml	**0.25ml**	**0.5 ml**	**0.0 ml**	**0.25 ml**	**0.5 ml**	
3 weeks old (N = 10)	**X±SD CV%**	176.7 ± 101.5 57.4	235.0 ± 185.7 79	251.6 ± 297.3 118.2	138.9 ± 134.3 96.7	116.4 ± 57.2 49.1	189.5 ± 173.1 91.4	0.902 0.487
5 weeks old (N = 10)	**X±SD CV%**	113.4 ± 182^{b} 160.5	16269.5 ± 493.9^{a} 39.9	16081.1 ± 416.6^{a} 39.9	115.9 ± 214.5^{b} 185.1	14364.2 ± 6408.3^{a} 44.6	14343.0 ± 6261.3^{a} 43.7	22.679 0.000
6 weeks old (N = 10)	**X±SD CV%**	168.3 ± 173.9^{b} 103.3	19093.0 ± 654.3^{a} 24.4	18581.8 ±4520.9^{a} 24.3	187.9 ± 246.2^{b} 131	21182.5 ± 4179.3a 19.7	18809.0 ± 3667.5^{a} 19.5	81.705 0.000
8 weeks old (N = 10)	**X±SD CV%**	192.7 ± 247.6^{c} 128.5	26537.8 ± 85.7^{ab} 11.6	27238.5 ± 1762^{a} 6.5	260.4 ± 286.7^{c} 110.1	22994.3 ± 4875.7^{b} 21.2	24741.4 ± 4059.8^{ab} 16.4	193.35 0.000
10 weeks old (N = 5)	**X±SD CV%**	995.6 ± 558.1^{b} 56.1	26365.2 ± 248.9^{a} 23.7	30085.2 ± 831.7^{a} 9.4	1360.6 ± 520.4^{b} 38.2	28194.5 ± 7686.1^{a} 27.3	32200.5 ± 2424.5^{a} 7.5	39.159 0.000
13 weeks old (N = 5)	**X±SD CV%**	571.2 ± 443.2^{b} 77.6	22692.8 ± 8876^{a} 39.1	25960.0 ± 198.1^{a} 8.5	767.4 ± 504.8^{b} 65.8	20615.4 ± 7680.7^{a} 37.3	25425.4 ± 8390.4^{a} 33	20.357 0.000

Note: ^{abc}Means within a row with different superscripts differ ($P < 0.05$).

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Table 4. Effects of administration routes and doses to ORT antibody titer after vaccinating with Ornitin Triple vaccine – Trial II

		Subcutaneous (SC)			Intramuscularly (IM)			F_{-value} P_{-value}
		0.0 ml	0.25 ml	0.5 ml	0.0 ml	0.25 ml	0.5 ml	
5 weeks old (N = 10)	X±SD CV%	198.9 ± 186.8 93.94	177.0 ± 132.3 74.73	197.0 ± 207.6 105.38	155.8 ± 88.0 56.44	196.9 ± 96.7 49.13	201.9 ± 80.5 39.85	0.164 0.975
7 weeks old (N = 10)	X±SD CV%	209.0 ± 262.0^{b} 125.34	25745.0 ± 5957^{a} 23.14	26458.0 ± 6798^{a} 25.69	270.1 ± 295.8^{b} 109.53	26125.0 ± 5388^{a} 20.62	26008.0 ± 5290^{a} 20.34	76.982 0.000
8 weeks old (N = 10)	X±SD CV%	224.7 ± 244.4^{b} 108.75	28669.0 ± 2005^{a} 6.99	29748.0 ± 2364^{a} 7.95	227.5 ± 234.3^{b} 102.99	27168.0 ± 2767^{a} 10.18	27985.0 ± 2665^{a} 9.52	520.28 0.000
10 weeks old (N= 10)	X±SD CV%	219.6 ± 222.4^{c} 24.41	24576.0 ± 2501^{ab} 10.18	27403.0 ± 1609^{a} 6.17	361.2 ± 286.9^{c} 79.43	23909.0 ± 4467^{b} 18.68	26746.0 ± 1150^{ab} 4.3	340.39 0.000
12 weeks old (N = 5)	X±SD CV%	1161.5 ± 706.5^{b} 60.8	39781.0 ± 8101^{a} 20.36	33063.0 ± 10749^{a} 32.51	821.0 ± 425.0^{b} 51.77	33788.0 ±10710^{a} 31.70	38160.0 ± 3462^{a} 9.07	32.767 0.000
13 weeks old (N = 5)	X±SD CV%	632.0 ± 233.0^{b} 36.91	28182.0 ± 5003^{a} 17.75	25839.0 ± 7878^{a} 30.49	672.0 ± 329.0^{b} 49.00	24533.0 ± 8752^{a} 35.67	25827.0 ± 3439^{a} 13.32	24.829 0.000

Note: ^{ab}Means within a row with different superscripts differ (P < 0.05).

Table 5. Effects of vaccination age to ORT antibody titer

	Dose of 0.25 ml		Df	T *P-value*	Dose of 0.5 ml		Df	t *P-value*
	21 Days old	35 Days old			21 Days old	35 Days old		
Vaccination day	175.7	186.9	35.69	-0.2703 0.7885	220.6	199.5	32.37	0.3319 0.7421
2 weeks post	15316.9^{b}	25935.2^{a}	37.29	-5.6364 0.0000	15212.1^{b}	26232.9^{a}	37.91	-5.7269 0.0000
3 weeks post	20137.8^{b}	27918.5^{a}	29.78	-6.8496 0.000	18695.4^{b}	28866.6^{a}	32.68	-9.5063 0.0000
5 weeks post	24766.5	24242.6	36.44	0.4164 0.6794	25990.0	27074.5	26.02	-1.3446 0.1904
7 weeks post	21654.1^{b}	36784.6^{a}	17.43	-3.8738 0.0012	31142.9	35611.3	11.06	-1.6732 0.1223
13 weeks old*	20403.4	26560.0	16.95	-1.4636 0.1616	25692.7	25833.1	18.00	-0.0545 0.9571

Note: ^{ab}Means within a row with different superscripts differ (P < 0.05); *11 weeks post for Trial I & 8 weeks post for Trial II.

sessed similarly to that of the injection routes. In trial I (Table 3), there was no difference between the injection dose of 0.25 and 0.5 ml of the same SC or IM injection route (P> 0.05). Trial II provided similar results to trial I (Table 4). It was clear from comparing results of both trials that the antibody titer of experimental chicken groups was not dependant on the injection doses up to 13 weeks old (Table 3, Table 4). This results were obtained only until 13 weeks, it is possible that there is simply not sufficient time to reflect a long of immunity response to higher injection doses (0.5ml vs 0.25ml). Therefore, for chickens raising in longer than 13 weeks, 0.5ml dose should be considered.

3.2.3 Effects of Vaccination Age to ORT Antibody Titer

The comparison of ORT antibody titer after vaccination between trial I and trial II was conducted by T test and the results were shown in Table 5.

These 2 trials was conducted on chickens of 2 different age groups (Table 5), trial I used 3-week-old chickens and trial II used 5-week-old chickens. According to the results of comparing ORT antibody titers of these 2 trials, it was found that the antibody titer after vaccination with the vaccine dose of 0.25 ml in trial I was lower than that of trial II at the times of 2, 3 and 7 weeks after vaccination (P < 0.05).

Likewise, at week 2 and 3 after vaccination with the vaccine dose of 0.5 ml, the ORT antibody titer of trial I was also lower than trial II (P < 0.001). However, after that and when the chickens reached the age of 13 weeks, no statistical difference was found between the trials (P > 0.05). As a result, it could be concluded that with the same

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vaccine doses, ORT antibody titer of 3-week-old chickes would increase at a slower rate compared to 5-week-old chickens; however, there would be no difference found between chickens of older age groups.

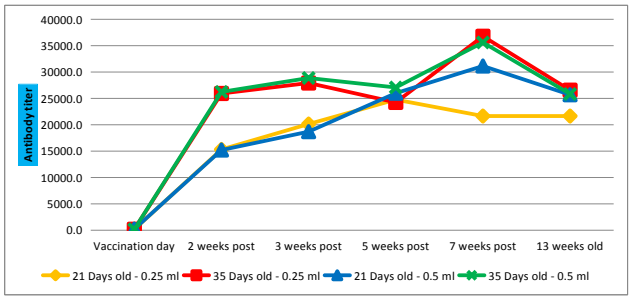

Figure 3. Effects of vaccination age and dose to ORT antibody titer

As recommended by vaccine manufacturer Ornitin Triple, the vaccine is suitable for turkeys 6 weeks of age and the appropriate vaccine dose is 0.5ml. However, there have been no specific recommendations for the local chicken breeds in Vietnam, which are raised for 13-16 weeks (turkeys are 20 weeks or more) and were especially susceptible to ORT at 5-6 weeks of age (Vo et al, 2019).

The appropriate age for vaccination of chickens was in accordance with the body weight, the level of completion of the immune system, health status, disease pressures and especially the effects of bacterial vaccines which often caused a strong reaction in vaccinated chickens (Nguyen Ba Hien et al, 2007).

Figure 3 showed that if chickens can wait until 5-week-old to be vaccinated would be better than 3-week-old chickens. However, if ORT outbreak happen about 5-6 weeks of age then chickens should be vaccinated at least 2 weeks before that, which is 3-week-old. Figure 3 also showed that for chickens raised less than 13 weeks, 0.25ml dose would be good enough, however, for chickens raised more than 13 weeks, 0.5ml dose should be considered.

3.3 Vaccine Reaction

3.3.1 General Body Reactions of Chickens

Body temperature

There was a fluctuation in body temperature after vaccination from day 5 of trial I and day 8 of trial II. There was a statistically significant difference between the control and vaccinated groups ($P < 0.05$). According to Nguyen Ba Hien et al, 2007, after vaccination, body temperature can increase by 0.5-1°C due to the immune responses that occur in the body. In this study, vaccination for younger chickens (3-week-old) would give a change in body temperature following the Ornitin Triple vaccination earlier (day 5 vs 8) than vaccination for older chickens (5-week-old) (Table 6, Table 7).

Table 6. Effects of administration routes and doses to chickens' body temperature – Trial I

	Subcutaneous (SC) (°C)			P F	Intramuscularly (IM) (°C)			P F
Dose	**0.0 ml**	**0.25 ml**	**0.5ml**		**0.0ml**	**0.25ml**	**0.5ml**	
1 days post	40.9 ± 0.4	41.1 ± 0.3	41.1 ± 0.3	**0.086 2.58**	41 ± 0.3	41.1 ± 0.4	41.1 ± 0.3	**0.3445 1.09**
2 days post	41.3 ± 0.3	41.4 ± 0.2	41.4 ± 0.3	**0.3 1.23**	41.4 ± 0.2	41.4 ± 0.2	41.3 ± 0.3	**0.4756 0.75**
3 days post	41.1 ± 0.2	41.2 ± 0.3	41.3 ± 0.4	**0.071 2.79**	41.2 ± 0.3	41.1 ± 0.3	41.2 ± 0.4	**0.7590 0.28**
4 days post	41.3 ± 0.2	41.3 ± 0.3	41.5 ± 0.3	**0.108 2.32**	41.3 ± 0.3	41.5 ± 0.3	41.4 ± 0.3	**0.4105 0.91**
5 days post	41.1 ± 0.4^{b}	41.4 ± 0.4^{ab}	41.5 ± 0.3^{a}	**0.013 4.75**	41.3 ± 0.2^{b}	41.5 ± 0.3^{a}	41.5 ± 0.3^{ab}	**0.0398 3.44**
6 days post	41.1 ± 0.3^{b}	41.4 ± 0.4^{a}	41.3 ± 0.4^{ab}	**0.035 3.59**	41.1 ± 0.2	41.3 ± 0.3	41.4 ± 0.5	**0.0642 2.90**
10 days post	41.7 ± 0.3	41.7 ± 0.3	41.7 ± 0.3	**0.735 0.31**	41.3 ± 0.2^{b}	41.7 ± 0.3^{a}	41.8 ± 0.3^{a}	**0.0000 14.58**

Note: ^{ab}Means within a row with different superscripts differ ($P < 0.05$).

Table 7. Effects of administration routes and doses to chickens' body temperature – Trial II

	Subcutaneous (SC) (°C)			P F	Intramuscularly (IM) (°C)			*P F*
Dose	**0.0 ml**	**0.25 ml**	**0.5ml**		**0.0ml**	**0.25ml**	**0.5ml**	
1 days post	41.6 ± 0.2	41.6 ± 0.2	41.7 ± 0.3	**0.503 0.70**	41.5 ± 0.2	41.6 ± 0.2	41.6 ± 0.2	**0.141 2.03**
2 days post	41.6 ± 0.2^{b}	41.7 ± 0.2^{ab}	41.8 ± 0.2^{a}	**0.005 5.92**	41.6 ± 0.3	41.6 ± 0.3	41.6 ± 0.3	**0.713 0.34**
4 days post	41.6 ± 0.2	41.7 ± 0.2	41.7 ± 0.3	**0.296 1.24**	41.6 ± 0.3	41.7 ± 0.2	41.6 ± 0.3	**0.580 0.55**
7 days post	41.5 ± 0.2	41.5 ± 0.3	41.6 ± 0.2	**0.084 2.59**	41.4 ± 0.4	41.6 ± 0.4	41.6 ± 0.3	**0.099 2.40**
8 days post	41.7 ± 0.2	41.8 ± 0.3	41.8 ± 0.3	**0.133 2.09**	41.7 ± 0.2^{b}	41.9 ± 0.3^{a}	41.8 ± 0.2^{ab}	**0.029 3.76**
10 days post	41.5 ± 0.2^{b}	41.8 ± 0.3^{a}	42 ± 0.3^{a}	**0.000 14.15**	41.5 ± 0.2^{b}	41.9 ± 0.2^{a}	41.8 ± 0.3^{a}	**0.000 11.85**
14 days post	41.6 ± 0.2	41.6 ± 0.2	41.7 ± 0.3	**0.876 0.13**	41.6 ± 0.2	41.7 ± 0.3	41.8 ± 0.3	**0.051 3.14**

Note: ^{ab}Means within a row with different superscripts differ ($P < 0.05$).

Feed intake

The effects of the vaccination to chickens' feed intake were evaluated in the same rearing environment in order to produce the most accurate result. The feed intake (g/chicken/day) after vaccination of each group was recorded every 2-3 days. The results showed that the feed intake of chickens in both trials decreased after vaccination. However, around 3-4 weeks afterwards, no significant difference was found (table 8 and 9).

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Table 8. Chickens' daily feed intake after vaccination – Trial I

Days old	Subcutaneous (SC) *(gam/bird/day)*			Intramuscularly (IM) *(gam/bird/day)*		
	0.0ml	0.25 ml	0.5 ml	0.0 ml	0.25 ml	0.5 ml
35* - 37	65.6	64.2	62.2	68.2	73.0	63.6
38 - 40	72.6	67.8	65.9	69.6	74.9	67.1
41 - 43	79.4	56.9	63.3	83.8	68.3	66.2
44 - 47	89.4	69.6	53.9	85.7	71.4	71.8
48 - 50	90.2	79.3	79.1	95.1	78.0	75.7
51 - 53	91.8	90.8	94.2	93.9	90.5	82.2

Note: *vaccination time

Table 9. Chickens' daily feed intake after vaccination – Trial II

Days old	Subcutaneous (SC) *(gam/bird/day)*			Intramuscularly (IM) *(gam/bird/day)*		
	0.0ml	0.25 ml	0.5 ml	0.0 ml	0.25 ml	0.5 ml
21* - 24	47.8	44.8	42.4	46.9	43.9	45.2
25 - 28	52.7	42.4	41.1	55.8	48.3	42.7
29 - 35	92.4	71.8	64.6	99.1	83.5	81.0
36 - 44	64.2	51.6	45.8	66.8	55.5	48.0
45- 49	73.0	67.6	59.2	75.9	63.4	62.5
50 - 52	98.4	95.6	89.4	92.3	89.5	94.5

Note: *vaccination time

3.3.2 Local Reactions at the Injection Sites

In the control groups, no reaction at the injection sites was recorded. In the vaccinated groups, swelling reaction started to increase 4-7 days after vaccination and peaked at 9-10 days after vaccination. In both trials, swelling reaction was found to develop the strongest in vaccinated groups with the dose of 0.5 ml by SC at neck and then 0.25 ml by SC at neck (Figure 4, Figure 5). In general, in both trials, IM route would produce a less severe swelling reaction compared to SC route; and at some time, the swelling at the injection sites of chickens with the vaccine dose of 0.5 ml would be more severe than those with the dose of 0.25 ml.

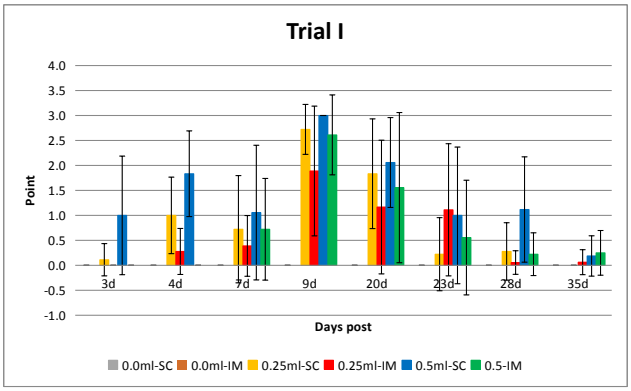

Figure 4. Effects of vaccine to swelling reaction at injection sites - Trial I

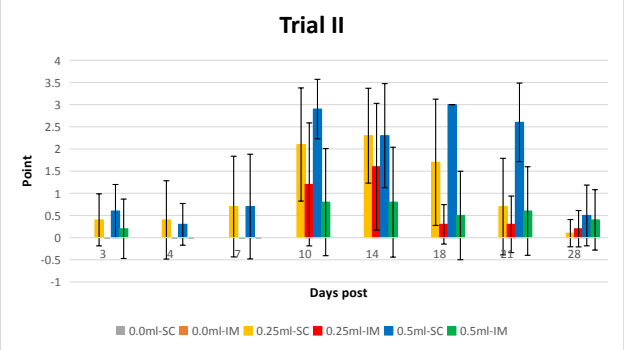

Figure 5. Effects of vaccine to swelling reaction at injection sites - Trial II

3.4 Growth Performance

The performance of chickens in each group was evaluated by their daily weight gain and the results were shown in Table 8 and 9. In both trials, within 2 weeks after vaccination, the chickens' performance had been influenced by the vaccine ($P < 0.001$), especially the SC-0.5ml group was most seriously affected. However later on (up to 91 days old), no difference was found between vaccinated groups and control groups ($P > 0.05$).

A comparison between groups with the same administration routes or the same vaccine doses was performed in order to compare the effects of administration routes or vaccine doses to the perfomance of chickens.

In both trials, at the beginning of the first 2 weeks after vaccination, all vacination groups had a significant weight gain lower than the control groups (Table 10, Table 11). At the same time, the first 2 weeks of both experiments, SC groups with the vaccine dose of 0.5 ml was documented to have a significant lower weight gain than those with the IM group with the vaccine dose of 0.25 ml ($P < 0.001$) (Table 10, Table 11). This could be the result of swelling reaction which was found to develop the strongest in vaccinated groups with the dose of 0.5 ml by SC at neck (Figure 4, Figure 5). The swelling reaction in the neck reduced feed intake especially during the first 2 weeks (Table 8, Table 9). Although, the vaccination groups did have an influence on chickens' performance at the beginning of both trials, however after two weeks, all vaccination groups recovered and no statistically significant influence on growth performance was documented ($P > 0.05$) (Table 10, Table 11).

4. Conclusion

In both trials, there was a development of ORT antibody titer by the 2 administration routes as well as with the 2 vaccine doses. The increase in antibody titer was quite stable in the vaccinated groups, which was statistically

Table 10. Chickens' average weight by age (week) – Trial I

Day-old	Subcutaneous (SC) (g/bird/day)			Intramuscularly (IM) (g/bird/day)			F_{-value} P_{-value}
	0,0 ml	0.25 ml	0.5 ml	0.0 ml	0.25 ml	0.5 ml	
20-35	29.8 ± 5.4^{ab} 17.99	24.3 ± 6.0^{cd} 24.61	20.4 ± 3.8^{d} 18.66	31.5 ± 5.1^{a} 16.24	26 ± 4.2^{bc} 16.07	23.2 ± 4.1^{cd} 17.55	13.000 0.000
36-49	31.3 ± 3.9 12.47	33.0 ± 4.4 13.43	31.4 ± 3.9 12.33	29.6 ± 8.0 26.88	28.6 ± 5.4 18.97	29.7 ± 4.3 14.53	1.6747 0.1475
50-63	30.9 ± 6.2 20.23	26.7 ± 5.2 19.44	30.9 ± 4.1 13.19	28.4 ± 7.9 27.68	30.4 ± 9.7 31.82	27.91 ± 7.4 26.53	0.5954 0.7035
64-91	33.3 ± 4.9 14.72	39.7 ± 6.6 16.73	35.3 ± 8.6 24.34	32.2 ± 3.5 10.7	35.2 ± 8.7 24.75	34.65 ± 5.2 15.13	0.2607 0.6123

Note: $^{a-d}$ Means within a row with different superscripts differ (P < 0.05).

Table 11. Chickens' average weight by age (week) – Trial II

Day-old	Subcutaneous (SC) (g/bird/day)			Intramuscularly (IM) (g/bird/day)			F_{-value} P_{-value}
	0.0 ml	0.25 ml	0.5 ml	0.0 ml	0.25 ml	0.5 ml	
36-49	30.7 ± 5.9^{a} 19.2	23.3 ± 5.7^{bc} 24.4	19.8 ± 7.9^{c} 40	30.3 ± 7.2^{a} 23.8	26.9 ± 8^{ab} 29.6	22.2 ± 6.8^{bc} 30.5	8.302 0.000
50-63	32.4 ± 5.6 17.3	34.6 ± 8 23.3	33.4 ± 7.3 21.7	31.3 ± 6.5 20.8	32.0 ± 12.4 38.6	31.3 ± 7.5 24	0.4887 0.7841
64-77	32.6 ± 9.6 29.6	29.2 ± 4.3 14.7	34.7 ± 8.6 24.7	28 ± 6.8 24.1	31 ± 4.3 13.9	28 ± 6.1 21.9	1.2812 0.2887
78-91	21.9 ± 5.6 25.7	24.6 ± 16.9 68.7	20.5 ± 3.8 18.5	26.4 ± 9.4 35.7	24.2 ± 5.1 20.9	23.7 ± 6.4 26.9	0.4469 0.8133

Note: ^{abc}Means within a row with different superscripts differ (P < 0.05).

significant compared to the control group.

There was no antibody titer statistically difference found between the two administration routes with the same dose and age (P> 0.05). ORT antibody titer of vaccine doses were different at the beginning but no difference when chickens reached the age of 13 weeks. Chickens vaccinated at 3 weeks old had a slower immune response than 5-week-old chickens.

Reactions at the injection site began to fluctuate 5-8 days after vaccination. The swelling reaction at the injection site by IM was less severe than SC route. Simultaneously, the smaller the vaccine dose, the less severe the swelling reaction.

Chicken growth index was affected 2-3 weeks after vaccination, however, no difference was found in later stages compared to the control group, which was reflected by feed intake and weight gain.

In conclusion, it was recommended that Ornitin Triple vaccine could be used for the young cross-breed coloured broilers in Vietnam at the dose of 0.25ml by IM or 0.5ml for older broilers which need to raise for more than 13 weeks. Moreover, it was also necessary to pay attention to the reaction at the injection sites after vaccination.

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ARTICLE

Clinical Evidence of Lyme Disease in Dogs and Disease Awareness Among Students and Veterinarians in Veracruz, Mexico

Blanca Paola Boria-Gamboa José Alfredo Villagómez-Cortés* David Itzcoatl Martiĺnez-Herrera

Facultad de Medicina Veterinaria y Zootecnia, Universidad Veracruzana, Veracruz, México

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ABSTRACT

Background: Lyme disease is a relatively new and zoonotic canine pathology mostly unknown by people involved in the management and care of dogs. Objective: to assess the knowledge about Lyme disease by veterinarians and veterinary students in Veracruz, Mexico. Methodology: three questionnaires were designed and applied to 290 individuals (40 small animal veterinarians, 50 in other professional practice and 200 veterinary students). Results and discussion: in general, the three groups were unaware about the disease, although there is a high interest in learning about this problem. Graduates from seven universities included in this study stated that Lyme disease was not part of the veterinary curriculum in their schools. Five clinical cases suggestive of Lyme disease occurring in dogs in the area were detected at the time of this research. Conclusion: although the presence of the agent causing Lyme disease has not been demonstrated in the studied area due to the lack of laboratory support and the apparent absence of the vector, it cannot be ruled out; furthermore, it is important that veterinary practitioners and student alike be aware of the potential presence of Lyme disease, paying particular attention to differential diagnosis when resembling clinical signs are observed.

1. Introduction

Lyme disease is a common zoonosis in the United States and Europe, where it can represent up to 90% of infections transmitted by ticks [1-4]. The disease is caused by Borrelia burgdorferii, which is transmitted by the bite of ticks that are usually parasite different animal species. The disease causes different signs affecting skin, joints, cardiovascular and central nervous system [5].

Mexico has identified the vector Ixodes in Baja California, the Yucatan Peninsula, the Gulf of Mexico and states in the northeast of the country. Out of 26 Ixodes species found in Mexico, only six have been identified in the state of Veracruz, without any proven act as vector of *Borrelia burgdorferi* [6]. It is possible that migratory birds infested with Ixodes carrying this germ can spread the problem [7-8], also, dogs that participate in national and international exhibitions can be a vehicle. In addition, although the presence of *Ixodes dammini*, the main vector of transmission demonstrated *B. burgdorferi*, has not be identified in the state of Veracruz, it is possible that other species of Ixodes or even other vectors may play a role in the spread of disease [6].

Some cases suggestive of Lyme borreliosis were de-

**Corresponding Author:*
José Alfredo Villagómez-Cortés,
Facultad de Medicina Veterinaria y Zootecnia, Universidad Veracruzana, Miguel Ángel de Quevedo Esq. Yañez, Col. Unidad Veracruzana, 91710, Veracruz, México;
Email: avillagomez@uv.mx

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scribed in the early 1990s in the Mexican states of Sinaloa and Nuevo Leon, but aetiological confirmation was not achieved [9]. In recent years, several positive people living in Mexico City, and in northern states such as Nuevo Leon, Tamaulipas and Coahuila were identified achieved [10]. Also, a 8.2% seroprevalence to Lyme disease was reported in dogs from Mexicali, along the border with the United States of America[11], and in Monterrey, Nuevo Leon, a seroprevalence of 1% in dogs was recognized by Meléndez Salinas *et al*. (2011) [12].

Lyme disease is considered a foreign animal disease in Mexico and there is no documented evidence of clinical cases in the state of Veracruz, because no investigation has been conducted. Therefore, the aim of this study was to determine the knowledge about Lyme disease that practitioners and students of Veterinary Medicine possess, as well as the possible existence of clinical cases suggestive of Lyme borreliosis in dogs of the Veracruz metropolitan area in Mexico.

2. Methodology

2.1 Location

The city of Veracruz is located along the coast in the central part of the state of Veracruz, Mexico and with the neighboring municipality of Boca del Río has over 600,000 inhabitants. The climate is tropical warm, with an average annual temperature of 26 °C and an average annual rainfall of 1500 mm. Most vegetation is of the rainforest type.

2.2 Study Design

To obtain the necessary data for this study, three different types of stakeholders who could provide information were considered, namely veterinary undergraduate students, small animal practitioners, and veterinarians in other professional fields. Private veterinary clinics were visited to inquire about clinical cases suggestive of Lyme disease. Also secondary information about the disease was acquired through literature review.

For each stakeholder type, a specific questionnaire was designed to obtain the necessary data. The questionnaire aimed to veterinarians tried to identify how well the veterinary profession was informed about Lyme disease and its biological cycle, years of clinical experience, the existence of clinical cases and its diagnosis and treatment, among other variables. The questionnaire aimed to the student community included variables such as: year of admission, species on which it has a predilection, definition of zoonosis, knowledge about Lyme disease as well as of the causative agent and vectors, clinical manifestations, affected species, treatment and interest about the disease.

2.3 Sampling

A convenience sampling was undertaked to collect information from veterinarians working in the Veracruz – Boca del Río metropolitan área. For veterinary undergraduate students, the local veterinary school was visited and students willing to participate in the study were selected. The veterinary medicine program emcompasses five years of education and every year 150 new students are admitted representing about 750 in total.

Each questionnaire was validated with a small number of members from each group before being applied to the whole sample

2.4 Data Analysis

Data were collected using a standardized form and were tabulated into a Microsoft Excel spreadsheet. Descriptive statistics were used to summarize information.

3. Results and Discussion

The study aimed to differentiate the knowledge and awareness existing about Lyme disease among various interest groups, namely undergraduates in veterinary medicine, veterinarias enrrolled in small animal practices and veterinarians devoted to other species and professional activities.

3.1 Students Survey

Out of 200 students surveyed, 98 were women and 102 men. Table 1 condenses information about them. The percentage of students aware of Lyme disease was calculated based on the number of students per class, not on the total respondents. Therefore, students who recently entered the university first had less awareness about Lyme disease than those than entered it later. This little awareness in the student community might result from the fact that Lyme disease is not a part of veterinary curriculum, so those who reported having some knowledge about the disease, have obtained it as a result of their personal curiosity.

Table 1. Sample composition of veterinary medicine students surveyed and their awareness about Lyme disease in Veracruz, Mexico

Class	Students, No. (%)	Students aware of Lyme disease, No. (%)
Fist year	18(9)	4 (4.44)
Second year	41(20.5)	5(24.4)
Third year	54(27)	5 (18.5)
Fourth year	45(22.5)	3(13.3)
Fifth year	42(21)	3(14.3)

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As in all academic activity, theoretical knowledge is the primary basis of students education. Out of 200 students only 20 (10%) are aware of Lyme disease. However, even if Lyme disease is considered an "exotic" disease which is not a public health problem today in Mexico, it is important to consider the risks that such zoonoses represent and take into consideration the potentially affected species, clinical signs, and the way of transmission, among other variables.

The value of information to the veterinary student community should be highlighted, since *Borrelia burgdorferi* is an agent which is important to identify, prevent and treat by the future veterinary professionals. Out of the 20 students who said they had information about Lyme disease and its characteristics, 16 people were right in terms of the causal agent, while the other four said that the agent is a rickettsia. In fact, Borrelia is a gram-negative bacterium, obligate anaerobe with periplasmic flagella that produce a spinning movement which allows the whole bacterium to move forward like a corkscrew [13]. Instead, Rickettsiae are, gram-negative and immobile anaerobes germs. Of this students, 16 (80%) are aware of the role of Ixodes tick as transmiting vector, while four students (20%) ignored it. The bacterium that causes Lyme disease, *Borrelia burgdorferi*, is present in other types of ticks, but it is only transmitted to humans and animals by Ixodes ticks. Vector of transmission in most of United States is *Ixodes scapularis*, the black-legged tick, while on the west coast of this country is *Ixodes pacificus* [14, 15]. In Europe the vector is *Ixodes ricinus* [16], in Australia *Ixodes holocyclus* [17], and in Asia *Ixodes persulcatus* [18].

Students were asked to mention at least four clinical manifestations of Lyme disease, whether in humans, dogs or other animal species which may be attacked by the causative agent. This question was only answered by 20 students with knowledge of Lyme borreliosis. Of these, eight (40%) mentioned the four clinical manifestations, five students (25%) cited only three events, five students (25%) cited only two, and two students (10%) only one.

Although students considered themselves to posess knowledge about the disease, some of them named clinical signs unrelated to Lyme disease pathology such as: acute attack to intestines, blood system blockage, spots on the skin throughout the body, "bone pain", epistaxis, jaundice and pyrexia, which shows that there is consistency between the opinion of the group of students who think they know about Lyme disease and have actual knowledge thereof. By the same token, when asked to name three species affected, out of 20 students who considered to be familiar with the disease, the affected species named were: dogs, cats, cattle, sheep, horses; bovine got the highest number of mentions. According to them, the numbers of affected species were: one species (10%), two species, 5 (25%), three species, 13 (65%).

In endemic areas of Lyme disease and surrounding areas, it has been noted that several species of domestic animals (dogs, horses and bovine cattle) are infected with *B. burgdorferi* [19, 20, 21]. In Nuevo Leon State, Mexico Lyme disease has been found in humans, dogs and deers [22, 23, 24], however the disease can be developed in other animal species because the infection with B. burgdorferi has been recognized in several other species all over the world.

Like in any other disease, in the case of Lyme disease is not only important to posess a general knowledge about the disease and its vector, but also about the treatments that could be administered early. This is critical because an early treatment allows for a complete cure while if treatment is not started right away, chances of total healing reduce. It is relevant to be informed of proper drugs to be used for each disease stage and the species concerned. Out of the 20 students who expressed to be aware of Lyme disease, 14 (70%) ignored treatment and only 6 (30%) reported to know about it. Of the latter, some students righthly mentioned some antibiotics such as doxycycline, penicillin, amoxicillin and ceftriaxone [25], but equally some respondents wrongly believe that the use of antiemetics and cefalexinas help cure the disease. Indeed, some students mentioned tick control or euthanasia as a treatment, even though this is not considered a proper treatment, but techniques to prevent the disease spreading.

As mentioned before, it is vital that after concluding their studies the new veterinary professional have the necessary knowledge on the different subjects related to veterinary medicine practice. From the perspective of the surveyed students, only 5% of them all believe that it is not necessary to acquire knowledge about Lyme disease, while most do.

3.2 Survey to Mixed Practice Veterinarians

Out of 50 veterinary doctors enrroled in mixed practice or dealing with animal species other than dogs and cats that participated in the present study, 72% (36) were men. The highest proportion of participants corresponded to male individuals in the range of 20 to 30 years. When considering the percentage of knowledge in relation to gender and age, the aforementioned group exhibited less awarenes about Lyme disease than veterinarians located in the 41 to 50 years bracket, who showed that they are more aware about the problem (66%). Female participants, despite representing a smaller proportion of the sample, showed a higher proportion of Lyme disease awareness (Table 2). Overall, of 36 men surveyed, only nine (25%) said they

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were aware about Lyme disease and of 14 women surveyed, 5 (36%) have notions on this condition. Regarding the professional experience, the majority of participants were in a range between 20-30 years (40%), although this age group showed less awareness than others; only three (15%) out of 20 exhibited understanding about Lyme borreliosis. In general, groups between 31-40 years and 51-60 years acknowledged to ignore everything about the disease. On the other hand, people in the 41-50 years and 61-70 years brackets said they had more information about the disease. As a result, although those with less knowledge of the subject are among the youngest age groups, the understanding of Lyme disease is not related to years of professional experience.

Table 2. Veterinarians enrolled in mixed practice or in other professional fields participating in the survey about awareness of Lyme disease in Veracruz, Mexico

Age, years	Professional experience, years	Sample composition			Awareness of Lyme disease	
		Male	Female	Total	Male	Female
20-30	0-6	12	8	20	0	3
31-40	0-15	3	3	6	1	1
41-50	0-25	6	0	6	4	0
51-60	0-30	9	3	12	1	1
61-70	15-46	6	0	6	3	0
Total		36	14	50	9	5

Most veterinary doctors paticipating in this survey were professors teaching diverse subjects in the local veterinary school, as well as some veterinary graduate students. Most respondents (45, 90%) were graduates from the Universty of Veracruz of which 12 (26%) were aware about Lyme disease; three (6%) were graduates of the Mexico´s National Autonomous University, of which two were aware of the subject (66%). There was also one graduate from the Mesoamerican University and one from the Higher School of Veterinary Medicine and Animal Science; only the veterinarian graduated from the later school had information on Lyme disease.

The number of species to which each veterinarian is dedicated seems to be related to the knowledge about the disease, because those who work with a greater number of species seem to have a better understanding of Lyme disease than those more specialized (Table 3). In relation to education, most respondents (36%, 18/50) have opted for a master's degree to support their professional work; 28% (14/50) have done so by continuous education courses, 18% (9/50) through two or more different training, while 14% (7/50) hold a doctorate. Professionals with specialization studies represented the smallest amount among the respondents, 4% (2/50).

Table 3. Animal species to which veterinarians enrroled in mixed practice are devoted in the Lyme disease survey in Veracruz, Mexico

Specie(s)	Number of veterinarians	Number aware of Lyme disease, No. (%)
Bovine	8	1(12.5)
Swine	4	1(25)
Poultry	2	0
Wildlife	2	0
Dogs and cats	8	37
Bovine and equine	3	66
Sheep and goats	4	0
Bovine and bees	1	0
Bovine, sheep and goats	4	25
Bovine, dogs and cats	3	33
Equine, sheep and goats	1	0
Equine, dogs and cats	1	0
More than four animal species	4	50
More than five animal species	5	60

Out of the 50 veterinarians enrroled in mixed practice or in other professional fields who collaborated in the study, 14 (28%) said they were aware Borreliosis, although four of them said they did not remember the name of the causative agent, while 72% (36/50) admitted they had no knowledge about it. As mentioned earlier, even though Borreliosis still does not represent a public health problem in Mexico, Lyme disease is considered as an endemic disease in many parts of the world. Gordillo-Pérez *et al*. (2003) investigated in 2346 sera the presence of *Borrelia burgdorferi* in Mexico City and northeast Mexico, detected by ELISA IgG antibodies against *Borrelia bugdorferi* and confirmed them by Western blotting. They found a seroprevalence of 3.43% in Mexico City and 6.2% in the northeast area of the country, being Tamaulipas the state with the highest seroprevalence [10].

As for the knowledge about the borreliosis transmission vector, out of 14 people who claim to posess knowledge about the disease, only nine have the notion that the genus Ixodes tick acts as a disease carrier. Unlike what was observed in the students survey, all veterinarians who responded knowing about the disease noted down the four clinical manifestations that were required in the survey about their knowledge on Borreliosis; For the purposes of such questioning, all veterinary doctors understand the clinical manifestations of the disease, however and in the same way as with the students, some clinical signs unrelated to the pathology were mentioned such as: jaundice, ascites, respiratory distress and pale mucous membranes.

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As for the questioning on the three species affected by the spirochete *Borrelia burgdorferi*, they were mentioned: human, deer and rat, except for an answer which wrongly included the bovine.

Regarding the adequate treatment of the disease, seven doctors correctly commented on tetracycline, while two others advised doxycycline. The same problem seen with students was repeated, since some people commented that as a part of treatment the vector should be controlled, while this is a preventive measure and not recovery one. Two other veterinary doctors are unaware of the treatment, while vaguely, one respondent commented that antibiotics should be used, to end the recommendation of use an "antiprotozoal such as sulfonamides or praziquantel" (sic).

Ninety-four percent (47/50) of the surveyed veterinarians were interested in knowing more about the disease and its importance for the veterinary community, while the remaining 6% (3/50) believe that this would not provide any benefit due that this disease is unrelated to the animal species they work with, and that it would only be important if the pathogen attacked their animal species of interest.

3.3 Survey Carried Out in Small Animal Veterinary Clinics

Out of 40 veterinary doctors dedicated to small animal clinics that participated in the survey, the majority (60%) were men (Table 4). Overall, a greater proportion of men (62.5%, 15/24) than women (56.25%, 9/16) said they were familiar with the disease. The largest number of respondents aware of Lyme disease were in the range between 41 and 50 years (89% 8/9) in the case of men, and in the category between 31 and 41 years (56 %, 5/9) in the case of women In relation to professional experience, as age and experience of participants increased it was observed a greater proportion of participants aare of Lyme disease.

Table 4. Veterinarians enrolled in small animal practice participating in the survey about awareness of Lyme disease in Veracruz, Mexico

Age, years	Professional experience, years	Sample composition			Awareness of Lyme disease	
		Male	Female	Total	Male	Female
20-30	1-5	8	2	10	3	2
31-40	3-18	3	9	12	2	5
41-50	15-27	9	5	14	8	2
51-60	25-35	4	0	4	2	0
Total		24	16	40	15	9

Participants in the survey were veterinary professionals graduated from six different universities, of which the majority (85%, 34/40) came from the University of Veracruz. Among these, the proportion of those who were aware of Lyme disease was 55.9% (19/34) (Table 5).

Table 5. Lyme disease awareness of veterinarians enrolled in small animal practice in Veracruz, Mexico based on their university of graduation

University of graduation	Graduated veterinarians, No.	Awareness of Lyme disease, No.
Benemeritous Autonomous University of Puebla	1	1
Mesoamerican University, Campus Puebla	1	0
Metropolitan Autonomous University	1	1
Zacatecas Autonomous University	1	1
Mexico´s National Autonomous University	2	2
University of Veracruz	34	19
Total	40	24

In relation to the professional update of veterinarians specializing in small species, most resort to continuous education courses (62.5%, 25/40), and to a lesser degree to diplomate programs (7.5%, 3/40), specialty training (2.5%, 1/40) or doctorate (2.5%, 1/40); while there is a good proportion (25%, 10/40) that opt for two or more of the above options. The lowest level of knowledge about Lyme disease understanding was found among veterinarians who opted only for the continuous education courses, despite representing the highest proportion of respondents.

Considering differential diagnosis of Lyme disease, small animal specialists mentioned: dilofiriasis, erlichiasis, anaplasmosis, among others. As a result, five doctors expressed seeing neurological alterations, uveitis and dermatitis in a single patient, and 14 selected three or more options, which included: severe lameness, neurological abnormalities, dermatitis, acute joint inflammation, uveitis and nephropathies; four clinicians reported myocarditis with arrhythmia, neurological disorders, dermatitis and uveitis, choosing two to three manifestations per patient. Consecutively to these manifestations, veterinary doctors determined as diagnosis in a higher percentage erlichiosis (18, 45%). Among the diseases not included in the questionnaire, the clinical doctors added that the manifestations were due to conditions such as: ligament injury (this in an animal which not only had severe lameness but also neurological alterations), mycosis, intervertebral discs disease, pyoderma, atopy, flea bite dermatitis, septicemia due to surgery, but especially conditions related to old-age. Canine anaplasmosis (5, 13%) was related to conditions such as neurological disorders, uveitis, dermatitis, acute joint inflammation and severe lameness. It is noteworthy that of the five veterinarians who made such a diagnosis,

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four said they knew about Borreliosis, four felt that their diagnosis was dilophilariasis, and three of them related the observed clinical signs to acute joint inflammation. There was no relationship between the conditions mentioned and Lyme disease was not considered in any of the patients, even in those clinics where it was said to have had Lyme disease cases in the past. Lyme disease manifests itself as a multi-inflammatory disease; that is, it affects the skin and spreads to the joints, causes abnormalities in the cardiac and nervous systems, and in its most advanced stage can affect other organs [26].

As mentioned before, out of 40 participants dedicated to small animal practice, 24 clinicians say they were aware of the disease (60%), but their knowledge was not applied in any diagnosis. Indeed, it is difficult to establish an early diagnosis of Lyme disease in animals. The characteristic skin lesion and chronic migratory erythema do not occur in dogs [5]; in adition, Lyme disease is not considered as an endemic disease in the study region, and thus is not usually considered in differential dignosis, even though some clinical signs are similar to other conditions, and the fact that the disease in canines can go for a long time without showing any signs.

About the vector, four veterinarians commented correctly about Ixodes tick; however, there is no certainty that *Ixodes dammini*, *Ixodes ricinus* and *Ixodes scapularis* exist in Mexico [6]. Montiel *et al.* (2007) reported the presence of a male, seven larvae and 15 nymphs of *Ixodes cookei* in the state of Veracruz in a Natural Protected Area at a height of 2420 masl, and in Nuevo León in the feces of *Urocyon cineroargenteus* and Peromyscus sp [27].

Out of the 40 participating veterinary doctors, five clinical cases suspected of Lyme disease were detected in two veterinary clinics, one located in the municipality of Veracruz which provided a case, and another clinic located in the neighbor municipality of Boca del Rio, which provided four additional clinical cases. Lyme disease clinically suspected cases were tested by a commercial diagnostic kit. One of the most confusing aspects in the Lyme disease diagnosis has been testing, since a positive result by itself does not constitute a diagnosis of Lyme disease; morover, the commercial kit is based on the ELISA serological technique, which provides the veterinarian with data regarding three more vector-borne diseases that affect dogs: canine erlichiosis, dirofilariasis and canine anaplasmosis [28]. The history of infection with ticks or contact with ticks, along with tick biting and suggestive clinical signs is of great importance to make a presumptive diagnosis of Lyme disease [29, 30]. Apparenty, it is easier to isolate the agent from skin lesions than from blood. Indirect immunofluorescence tests with sera conjugated with IgM and IgG are widely used [31]. Patients with late disease manifestations (arthritis, cardiac or neurological abnormalities) show elevated IgG titers [32]. Indirect ELISA test was more sensitive and specific than the immunofluorescence test [33]. In Mexico, the presence of Ixodes ticks has not been established yet and laboratory tests for Lyme diasease such as Wester blotting are not extensively available in veterinary clinics, as a consequence it cannot be determined with total certainty that suspected clinical cases are positive, since with available diagnostic tests a positive result may be due to cross reactions.

All veterinarians enrolled in small animal practice in Veracruz, Mexico want to know more about Lyme disease and consider more information about this topic should be shared with the veterinary community. In study conducted in British Columbia to determine physicians' level of awareness and knowledge of Lyme disease in a low-prevalence area, Henry *et al*. (2012) found that physicians were knowledgeable about the clinical signs and symptoms of Lyme disease and aware of the risk of the disease despite being in a low-endemic area and suggested that raising awareness among physicians that Lyme disease is reportable might improve reporting of future cases [34]. As Cripps (2000) states, this situation may be exacerbated where there is inadequate communication between veterinarians, the various health care professionals and public health organisations. Undergraduate and postgraduate training courses must promote a greater understanding of the importance of zoonoses and of how to investigate and control them. A huge area of oportunity is perceived to increase awareness amongst qualified veterinary personnel and human health care workers and to facilitate inter-disciplinary discussions and collaborative ventures [35]. The re-emergence of zoonoses, together with other issues such as bioterrorism, pollution incidents,antimicrobial resistance, xenotransplantation, among other factors, make urgent a collaborative interprofessional approach to veterinary public health [36].

In conclusión, awareness of Lyme disease is almost null among the veterinary student community and veterinarians enrolled in mixed practice or in other professional fields, but they are open to be educated on Lyme disease. All veterinarians enrolled in small animal practice showed interest in learning more about Lyme disease and stressed the importance of managing adequate information within the veterinary profession, but none included it in their differential diagnosis of suspected patients, even when signology in some sick animals is consistent with Lyme disease.

In the region of Veracruz, Mexico there are not official reports on presence of Lyme disease and its vector; thus, this disease is not included in any subject of the current

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curriculum at the local School of Veterinary Medicine. Since five cases suggestive of borreliosis in dogs were determined based on clinical examination and ELISA testing, it is suggested to carry are more studies to confirm the presence of Lyme disease and its vector, to educate veterinary students and vererinarians alike on the disease, and to review the curriculum oft the local Veterinary Medicine School to increase awareness.

Conflict of Interest

The authors declare that they have not any conflict of interest.

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REVIEW

Epidemiology and Control of Congo Fever in Sacrificial Animals of Pakistan

Hafiz Muhammad Rizwan[1*] Muhammad Sohail Sajid[2] Haider Abbas[1] Muhammad Fiaz Qamar[3] Qaiser Akram[4] Mahvish Maqbool[2]

1. Section of Parasitology, Department of Pathobiology, College of Veterinary and Animal Sciences, Narowal, Sub campus UVAS, Lahore
2. Department of Parasitology, University of Agriculture, Faisalabad, Pakistan
3. Department of Pathobiology, College of Veterinary & Animal Sciences, Jhang, Sub campus UVAS, Lahore
4. Section of Microbiology, Department of Pathobiology, College of Veterinary and Animal Sciences, Narowal, Sub campus UVAS, Lahore

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ABSTRACT

The cases and deaths due to Crimean-Congo haemorrhagic fever (CCHF) virus commonly known as Congo virus (fatality rate 15%) have been reported throughout Pakistan from the last few years especially during religious occasion, Eid-ul-Azha. The annual increase in death rates due to CCHF demonstrate the importance of awareness of Congo fever at academia as well as public level. The symptoms of Congo fever which appear one to nine days after tick bite, include sudden high fever, muscle aches, abdominal pain, headache, dizziness, sore eyes, jaundice, mood swings, confusion, aggression, and sensitivity to light. The other signs include sore throat, joint pain, vomiting, diarrhea, hemorrhages, and bleeding from skin and large intestine. The Infection has been reported in many species of wild as well as domestic animals including hares, cattle, sheep, goats, dogs, mice and hedgehogs. At least 31 species of *Hyalomma, Boophilus, Rhipicephalus, Dermacentor* (Ixodidae: hard ticks) act as vector of CCHF in which transovarial, transstadial and venereal transmission occurs. The virus attacks the immune system of the host and influences the immune cells. The Congo fever virus can be isolated from blood, plasma and many body tissues (kidneys, liver, spleen, lungs, brain and bone marrow). Mice inoculation, enzyme-linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR) can be used for detection of the infection. Furthermore, IgM and IgG antibodies against CCHFV can also be detected and quantified. Education of general public, tick control with acaricides, use of anti-CCHFV immunoglobulin, usage of approved repellents to prevent tick bites, wearing neutral-coloured garments, application of a permethrin spray to the clothing, avoiding tall grasses and shrubs, applying sunscreen, avoiding direct contact with the blood or tissues of animals are the factors for successful prevention of the infection.

**Corresponding Author:*
Hafiz Muhammad Rizwan,
Section of Parasitology, Department of Pathobiology, College of Veterinary and Animal Sciences, Narowal, Sub campus UVAS, Lahore;
Email: dr.hmrizwan@yahoo.com, hm.rizwan@uvas.edu.pk

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1. Introduction

Crimean Congo hemorrhagic fever (CCHF), one of the tick borne, zoonotic viral infections, is significantly fatal, causing causalities in different areas of the world; particularly in developing countries. The CCHF is caused by Nairovirus which belongs to the family Bunyaviridae. Pakistan, being developing country faces many challenges due to communicable and non-communicable diseases over the period of time. In Pakistan, hasty climate change triggered by occupational and industrial activities to upkeep the human population has been measured the most paying basis for emergence or re-emergence of CCHF. The CCHF is mainly prevalent in southeastern Europe, Africa, Middle East and Asia. In general, the vectors of Congo fever are hard ticks of Ixodidae family, including *Hyalomma (H.)* (particularly H. *marginatum*), *Boophilus, Rhipicephalus* and *Dermacentor*. The ixodid ticks are also notorious as the main reservoir for CCHF. Almost 30 Ixodid species have been found positive for CCHF through screening [21]. Congo fever can be transmitted to the human through vector bite, direct contact with tissues, blood of infected livestock and body fluids of infected patients. The nosocomial transmission, contact with viremic livestock and/or human blood and sporadic outbreaks due to tick bite have been reported frequently in Pakistan.

The farmers and health care workers are at high risk of CCHF. Clinical symptoms related with CCHF are haemorrhage, fever and sometimes nonspecific prodromal symptoms may occur. Differential diagnosis of CCHFV is somewhat difficult from viral haemorrhagic fevers (VHFs) and undifferentiated febrile. It is epidemic with high case fatality ratio (3-30%) du to hindrances in prevention and treatment. The case fatality rate of CCHF up to 5-80% has also been reported [27].

The prevalence of CCHF has been reported from different areas of the world including South Africa, Tajikistan, India, United Arab Emirates, Iran, Turkey and Pakistan [4,6,12,33,34,45,47,48]. Pakistan is at 4th position after Turkey, Russia and Iran in infected cases and 2nd after Turkey in fatal CCHF cases, respectively according to The Program for Monitoring Emerging Diseases (Pro MED) during 1998-2013 [23].

The domestic and wild animals including goats, cattle, birds and rodents act as amplifying host for CCHF virus (WHO, 2013). It is considered as emerging arboviral infection due to climate change resulting in increased vector bionomics. Till now, there is no availability of commercial vaccine and specific treatment against this virus [3]. The climatic conditions present in Pakistan are found to be suitable for vector growth and replication so chances of CCHF spread has increased many folds [26]. In Pakistan, first case of Congo fever was reported in 1976 in General Hospital, Rawalpindi [6] and later on, it becomes endemic in year 2000 [51]. In Pakistan, public health sector has been facing challenges by many epidemics of Congo fever and still it is going on. At present polio, dengue epidemics and CCHF emerged as important fatal infection from public health point of view.

Epidemiological profile of CCHF is reported from different areas of the world [44,46], but unfortunately in Pakistan, no data is available regarding this. National Institute of Health (NIH) is the only institute in Pakistan which in collaboration with WHO monitoring CCHF on daily bases and providing instructions to the concerned health department [20]. About 20 causalities were reported from Pakistan in 2016 due to CCHF and high prevalence was reported in Sindh [11] due to lack of proper monitoring system for CCHF outbreak in Pakistan [26]. Therefore, main objective of this review is to discuss the factors responsible for transmission of Congo fever due to which Pakistan is at high risk for CCHF and to check the epidemiology and control measures against CCHF.

2. Transmission of CCHF

Main vectors and reservoirs for CCHFV are hard ticks. Due to more prevalence of *Hyalomma* vector, wide range of animals can act as host for CCHFV which help in hematophagy for different tick stages. The favorable climatic and ecologic conditions, arid types of vegetation and public behavior are the potent factors which affect the establishment and maintenance of CCHF in an area. Larval and nymphal stages of *Hyalomma* genus feed on hares and small birds while adult stages feed blood on sheep, cattle and other large mammals. There are some other species which are three-host ticks and after each molting, they drop off from their host. Migration of infected animals from infected area to other area is the main factor for CCHF transmission. Transmission can occur through contact with infected animals' blood and other products, person to person contact and contact with infectious body fluids. Humans may participate in increasing in CCHFV transmission through recreational activities, change in use of land and through migration of infected animals [30]. Due to such kind of activities, there may be increase in incidence of CCHF in future.

Main route of CCHF transmission is tick bite, other than this direct contact with infected animal blood, tissues and body fluids and skin of infected person my play important role for the transmission of the disease [16,40]. Mostly, disease is asymptomatic in animals [14] and

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is considered a threat for population including farmers, veterinarians and health care workers dealing with CCHF infected persons. There is another type of viral transmission occurred in which requirement of viremic animal is not compulsory and direct transmission takes place during feeding of infected ticks to the others. During this, feeding viral substances present in the tick saliva increases the viral transmission. Due to inefficient managemental practices and biosafety policies, chances of CCHF epidemics increased [51].

In Pakistan, majority of people are interrelated with agriculture sector, especially in rural areas and are engaged with livestock management. Numerous vectors acting as the main source of pathogen transmission are more prevalent in agricultural areas of Pakistan, so, community are at high risk for vector-borne diseases especially CCHF. Across boundaries, livestock movement also play important role in transmission of CCHF [22]. In Pakistan, Khan et al. [25] reported that about 37%, 45% and 18% cases of CCHF transmitted by person to person, animal contact and unknown ways, respectively. There are some risk factors which play important role in transmission of CCHFV i.e. crushing and rubbing of infected ticks on skin and slaughtering of infected animals and nosocomial infection. Factors responsible for the transmission of CCHF in animals and humans are given in figure 1.

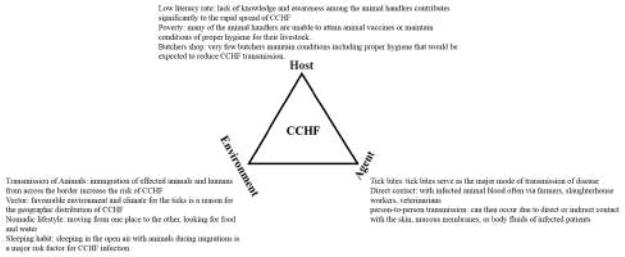

Figure 1. Factors responsible for the transmission of CCHF in animals and humans

Humans can get infection through tick bite or bare skin contact with tick, per mucosal and percutaneous route. Aerosol transmission is also considered a possible route in some territories of Russia but there is no sure report [40]. There is a single report regarding sexual transmission [28] but it is necessary to confirm the presence of CCHFV in body fluids of livestock workers and slaughterhouse workers as they are at high risk of sudden exposure to the virus [3]. Transmission of CCHF is also reported through human to human interaction and 15-20% mortality rate reported in health care workers due to direct contact with body secretions of infected patient [5,13,41]. Due to lack of early diagnosis facilities, chances of nosocomial transmission increased many folds and along this needle prick injuries and splash also reported due to lack of personal protective equipment supply [15].

3. Epidemiology of CCHF

People working with animals i.e. livestock workers, slaughterhouse workers and animal herd workers and travelers in endemic arears are at high risk for CCHF. It is also reported that health care workers are at risk through contact with infected body fluids and blood.

The CCHF first described by a Tajikistan physician in 1100 AD in a patient showing haemorrhaic symptoms [31,43]. Disease was first identified during an outbreak in 1944 in Crimea and later in 1965 identified and isolated in Congo and so the name Crimean-Congo haemorrhagic fever (CCHF) was given. Disease is endemic in many areas of the world i.e. Middle East, Africa, Asia and Eastern Europe (Messina et al., 2015). Among tick-borne diseases, CCHF covers a large geographical area and virus has been isolated from 30 countries covering four regions Asia (Tajikistan, China, Afghanistan, Pakistan, Kazakhstan, India, Uzbekistan), Middle East (Iraq, United Arab Emirates (UAE), Iran, Oman Kuwait, Saudi Arabia), Africa (Mauritania, Democratic Republic of Congo, South Africa, Uganda, Sudan, Nigeria, Senegal) and Europe (Kosovo, Russia, Turkey, Bulgaria, Spain, Greece) (WHO, 2016). Due to its widespread distribution, it must be considered as global health threat. Distribution of CCHF is related with prevalence of ixodid ticks especially *Hyalomma* genus. In Europe, main vector for CCHF is *H. marginatum* while in Asia, *H asiaticum*. In 2006, *H. marginatum* was first time detected in southern Germany and Netherland [24,32]. In January 2011, first case of CCHF was reported in India and main vector involved in transmission was *H anatollicum* [35].

Its name Congo, is the main source of confusion about its prevalence outside the Congo, however, it is reported from Pakistan by a senior surveillance coordinator of communicable diseases in Islamabad, Dr. Muhammad Najeeb Khan Durrani. In Pakistan, CCHF virus was isolated very first time in 1960s from ticks collected from Changa-Manga forest, Lahore [9]. Asia-1 and Asia-2 genotypes of CCHFV have been considered as the most prevalent genotypes in Pakistan. However, the first case was reported in 1976 in Central General Hospital, Rawalpindi. During this year total eleven cases were reported and one infected patient was died among them, however, one surgeon and an operation theatre attendant were also died due to direct contact with patient [12]. In 1994, cases and death due to CCHF was reported from Quetta, Baluchistan [19] and in 2000 from Khyber Pakhtunkhwa [8], in 2002 from Bagh in Azad Jammu and Kashmir [6]. Number of deaths reported in last six years from different provinces of Pakistan is given in figure 2. World Health Organization along with the Na-

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tional Focal Point, Ministry of Health, Pakistan reported 26 cases in 2010, 61 cases in 2012, 16 in 2013, 25 in 2015 [37,50]. The percentage of confirmed CCHF cases reported in Pakistan during different seasons is given in figure 3.

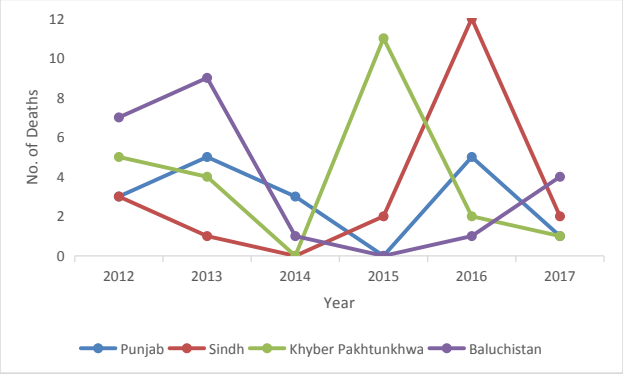

Figure 2. Number of deaths reported from different provinces of Pakistan during 2012 to 2017

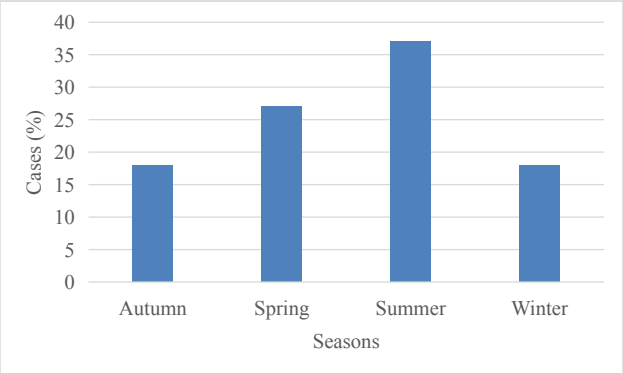

Figure 3. Percentage of confirmed CCHF cases reported in Pakistan during different seasons

Change in climatic conditions, worldwide distribution of *Hyalomma* vector and availability of range of hosts in European countries especially Mediterranean Sea areas are more prone to CCHF infection in near future. Dry climates, arid vegetation and prevalence of large and small mammals favor the *Hyalomma* growth by providing hematophagy for different life cycle stages. Human activities and ecological factors are important for establishment and maintenance of CCHF in an area. Humans can minimize the CCHF risk through modifying their recreational activities, land use and by adopting the policies for safe transportation of animals [30].

4. Livestock and Eid-ul-Azha

Livestock play an important role in the economy of Pakistan, owned by rural community and commercial dairy farmers. In Pakistan, currently, 24.24 million sheep, 23.34 million buffalos, 22.42 million cattle and 49.14 million goats population have been reported [1]. A large number of community is directly linked with these animals such as veterinary care, tick removal, animal slaughtering and butchery. So, chances of CCHF transmission increase from animals to humans as reported in a study from Afghanistan [36].

Unfortunately, in Pakistan, people of high-risk areas are in close contact with the livestock that increases the CCHF risk. Animal transportation from one region to other also help in transmission of CCHF especially at Eid-ul-Azha (a religious festival of Muslims to sacrifice cattle to give away to others). As a result, large number of CCHF cases are reported around that time [29,42]. Eid-ul-Azha is celebrated according to the Islamic calendar and now a days, it is conceding with summer season when environmental conditions are suitable for tick growth, development ultimately increases tick population carrying CCHF virus [2]. Movement of animals from rural areas to urban areas increases CCHFV transmission to the humans. Due to lack of polices for prevention against CCHF, number of cases are increasing day by day.

Before few days of Eid-ul-Azha, it is a tradition to keep animals at houses and this increases the chance of transmission through the bite of the infected tick. Mostly people slaughter animals by themselves or appoint a butcher at their homes, so, during slaughtering chances of transmission also increases [38]. Furthermore, butchers also act as source of CCHF transmission by providing services from home to home. Another major problem is the waste material of slaughtered animals, which is kept in open places and there is no proper disposal system.

Besides these sacrificial activities, accidental death of animals is also a major source of transmission. Animals are kept on roads or open places for weeks and lack of proper disposal system also aggravate the condition [7]. Although, the Ministry of National Health Services Pakistan has started an awareness scheme and regarding this, a letter has been issued in June 2015 focusing on preventive measures against CCHF. But unfortunately, it did not describe any guidelines for slaughtering and animal movements across boundaries [2].

Special precautions must be taken while buying the sacrificial animals like inspection of the animal body for ticks, each day and avoidance from crushing of ticks with bare hands. Do not try to remove the tick with your fingers. Wear light colored, full sleeve clothes and shoes with socks. Apply an insect repellent on the exposed area of body. Take a shower and change your clothes as soon as you return home.

5. Prevention and control

Difficulty in the control of CCHF infection is due to un-

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noticed transmission of virus in domestic animals during tick-animal-tick cycle and further due to wide range of tick vectors. Due to unavailability of vaccines against these vectors, acaricides are the only option for control of these vectors at livestock farms. So in this scenario, public awareness regarding this disease and its vector is the only way to reduce the burden of exposure to CCHF.

One of the primary reasons for the spread of CCHF is the lack of reasonable facilities and care center to accommodate quality care for animals. Proper monitoring of animals which imported from other countries or endemic regions, especially during Eid ul Azha, should be done to control CCHF. One of the best ways to reduce the transmission of CCHF virus from animal to human is the use of gloves and proper protective clothes. Try to avoid direct contact with infected person and infected body fluids. Media should provide awareness regarding symptoms, route of transmission and preventive measures against CCHF i.e. avoid direct contact with tick, and use proper clothing during animal cleaning, use spray to protect your animals against ticks, avoid backyard animal slaughtering practices, cover mouth and nose during start of slaughtering, slaughtering should be done in abattoirs or restricted places. If hands are blood stained, do not rub eyes or nose. The offal and leftovers of the animal should be disposed-off properly. Slaughtering area should be washed and cleaned after the sacrificial activities.

Besides these awareness programs, informative lectures, training workshops are also necessary to properly educate the health care professionals. Government should also play its role by implementing the strategies and providing financial resources to the research institutes for development of vaccines against livestock and agricultural infectious diseases. During the religious occasion of Eid-ul-Azha, it is necessary to provide suitable diagnostic facilities and quarantine measures to help in prevention of the disease spread and reduction in the causality rate due to CCHFV. There is need to provide registry and tracking system for monitoring of animals and people from endemic areas to other provinces and across the country borders. Epidemiological survey should be conducted to get familiar with the prevalence of tick fauna and other associated factors which can aggravate the condition by providing suitable environment and transmission route for vector-borne diseases. Environmental hygiene and personal protection are also the essential components of prevention.

6. Conclusion

Crimean-Congo hemorrhagic fever is highly prevalent disease across the world and Pakistan is at high risk due to higher abundance of ticks and suitable environment for their growth. Flaws in health care system policies, high prevalence in rural community engaged with livestock rearing and association of livestock with Eid-ul-Azha also increase the risk of CCHF. Moreover, workers in diagnostic laboratories are also at risk to develop infection through contact with viral samples during routine dealings. For all these reasons, Center for Disease Control and Prevention has characterized CCHFV as a BSL-4 pathogen. Some of these factors belong to culture and some are physical, so, they should be dealt in different ways. It is suggested to devise some polices to educate the community about CCHF and its transmission routes. This will be helpful for minimizing the risk factors and will help to stop the nosocomial transmission.

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ARTICLE

Ingestive Behavior and Nutritional and Physiological Parameters of Sheep Fed Diets Based on Cashew Byproduct

Clésio dos Santos Costa[1] Tallita da Ponte Ribeiro[2] Roberto Cláudio Fernandes Franco Pompeu[3] Fernando Lisboa Guedes[3] Marco Aurélio Delmondes Bomfim[3] Arnaud Azevedo Alves[4] Luciana Freitas Guedes[3] Delano de Sousa Oliveira[2] Marcos Cláudio Pinheiro Rogério[3*]

1. Department of Animal Science, Federal University of Ceará, Fortaleza, Ceará, Brazil
2. Department of Animal Science, State University Vale do Acaraú,, Sobral, Ceará, Brazil
3. Brazilian Agricultural Research Corporation, Embrapa Goats & Sheep, Estrada Sobral-Groaíras, Km 04, 62.011-970, Sobral, Ceará, Brazil
4. Department of Animal Science, Federal University of Piauí, Teresina, Piauí, Brazil

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ABSTRACT

Objective of this study was to evaluate the ingestive behavior, feed efficiency, and nutritional and physiological parameters of sheep that were fed diets based on byproducts from the processing of cashew. The experiment was conducted in a completely randomized design with a 4 × 2 factorial arrangement with four levels of inclusion (6 %, 11 %, 16 %, and 21 % of cashew byproduct) and two forms of processing—with chemical treatment (CT) and without chemical treatment (NCT). The interaction levels of inclusion of the byproduct of cashew versus chemical treatments was not (P>0.05) for the dry matter intake, consumption of organic matter. No effect was observed (P>0.05) for the intake of dry matter in function of the type of chemical treatment used in the byproduct of cashew. There was no effect of interaction (P<0.05) between levels of inclusion and chemical treatment applied or not on the byproduct of cashew for the coefficients of digestibility of dry matter, organic matter and crude protein (P>0.05). The inclusion of the byproduct of cashew did not influence the behavioral parameters, intake and digestibility of the diets of sheep, being recommended to use up to the level of 21%.

1. Introduction

Raising sheep in the Northeast region of Brazil is of great social and economic relevance for to supply meat that is easily accessible/available to rural populations and populations at the peripheries of large cities. In this region, forage production shows strong seasonal variation mainly because of the poor distribution of rainfall and long periods of drought. Combined with low forage production, the seasonality of rainfall has a strong influence on the production of green mass per hectare and the nutritional value of fibrous sources of fodder provided

**Corresponding Author:*
Marcos Cláudio Pinheiro Rogério,
Brazilian Agricultural Research Corporation, Embrapa Goats & Sheep, Estrada Sobral-Groaíras, Km 04, 62.011-970, Sobral, Ceará, Brazil;
Email: marcos.claudio@embrapa.br

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to ruminants. This explains, in part, the sharp decrease in the productivity of herds of ruminants in traditional systems, particularly in periods of drought [1].

In an effort to reduce input costs, the use of agroindustrial byproducts to replace fiber sources in the diets of ruminants is increasing owing to an increase in the production of the national fruit. However, these byproducts have a high content of polyphenolic compounds that hinder the utilization of nutrients by animals [2].

Urea application is widely employed to increase the bioavailability of nutrients and improve the nutritional value of byproducts [3,4], however, its effects on animal behavior are still unknown.

The addition of urea to byproducts modifies the ingestive behavior of animals, as it is correlated with the physical and chemical characteristics of food and consequently the transit time of digestion, motility of the pre-stomachs, and feed grinding fineness and power level required. Chewing, in turn, is related to the size of the particles that reach the rumen and this interferes with the digestion of food and consequently influences consumption [5]. Rumination time is influenced by the nature of the diet and is proportional to the cell wall content of the byproduct in the feed [6]. Thus, the assessment of eating behavior, composed of the time of feeding, rumination, and leisure and efficiencies of feeding and rumination, can assist in evaluations of diets and allow the adjustment of food management for improved productive performance [7].

The objectives of this study were to evaluate the ingestive behavior, feed efficiency, and nutritional and physiological parameters of sheep that were fed diets based on byproducts from the processing of cashew, with and without the addition of urea.

2. Materials and Methods

The experiment was performed from March 22 to April 22, 2006, in the sector of animal digestibility, at Vale do Acaraú, experimental farm, Universidade Estadual Vale do Acaraú - UVA, in Sobral, Ceará. The physiographic zone of the Sertão region of Ceará is 3º36′S, 40º18′W, at an altitude of 56 m.

The region has BSh type climate (Köppen classification), dry weather, and receives approximately 888.9 mm during the rainy season (January to June), corresponding to 92.6 % of the total yearly rainfall. The annual maximum, average, and minimum temperatures are approximately 33.3, 26.6 and 22.0 °C, respectively, and the annual average relative humidity is 67.9 %.

The agroindustrial byproduct of cashew (*Anacardium occidentale* L.) was obtained from Cajubrás-SA, located in Pacajus-CE and was composed of bagasse of cashew pseudofruit after extraction of the juice and drying in the sun. The hay was prepared from Aruana grass (*Panicum maximum* "Aruana"). The cotton pie and maize grain were obtained from Sobral in sufficient quantity for the realization of the entire experiment.

The cashew byproduct was treated with urea before the start of the experiment. The amount of urea added was calculated as 5 % of the weight of the dry byproduct (5 kg urea per 100 kg byproduct) diluted with water at a 1:4 ratio. The cashew byproduct was treated by distributing the urea solution using a watering can, then covered with plastic for 20 days, without adding a urease source [8].

The experiment was conducted in a completely randomized design with a 4 × 2 factorial arrangement with four levels of inclusion (6 %, 11 %, 16 %, and 21 % of cashew byproduct) and two forms of processing—with (CT) and without chemical (NCT) treatment. Three animals per treatment were used, and the experiment lasted 34 days, with 27 days corresponding to the period of adaptation (when the leftovers were between 15 % and 20 %) and seven days for collection. The supply of the ration was divided over two feeding times (08:00 and 17:00).

For the determination of consumption, food and orts were collected before the supply during the collection period; moreover, feces were collected and aliquots of 20 % of the total weight were weighed, stored in plastic bags, and stored at -20 °C. At the end of the experiment, the material was thawed and ground to pass through 1 mm sieve for chemical analysis.

For determination of dry matter (DM), organic matter (OM) and ash, ether extract, and crude protein (CP), the methodology proposed by [9] was followed. Quantification of the neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose, hemicellulose, and lignins was performed according to the methodology proposed by [10]. These analyses were performed at the Center of Agricultural and Biological Sciences of the Universidade Estadual Vale do Acaraú (Sobral - Ceará) (Table 1, 2).

For calculation of the TDN of the experimental diets, we used the equation recommended by the [11]. The percentage of total carbohydrates (CHOT) was calculated using the equation proposed by [12] and that of non-fibrous carbohydrates (NFC) was calculated using the equation suggested by [13]. The coefficients of digestibility of DM, OM, CP, and NDF were determined using the following formula: [(consumption of nutrient in grams - quantity of the nutrient in feces)/consumption of nutrient in grams]/100 [14].

The collection of ruminal fluid for measurements of ruminal pH in 4 pre-established times (0 h or prior to the delivery of the diet, 2, 5, and 8 h postprandial) was per-

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formed using an esophageal probe. The pH was measured immediately after the collection of ruminal fluid. Samples of approximately 50 ml of rumen fluid were acidified in 1 ml of sulfuric acid (1:1) and stored at -5 °C for future analysis of N-NH_3, which were carried out at the Laboratory of Animal Nutrition, Universidade Estadual Vale do Acaraú -UVA.

Ammoniacal nitrogen in the rumen fluid was determined by distillation with magnesium oxide, using boric acid containing a mixed indicator of methyl red and bromocresol green and titrating with 0.1N HCl.

Blood collection was performed by puncturing the jugular vein at the same time as the ruminal fluid was collected. The dosage of urea was measured using Bioclin kits at the Laboratory of Animal Nutrition, Universidade Estadual Vale do Acaraú -UVA and Bromatology Laboratory of the Center of Technological Education (CENTEC - Unidade Sobral).

The behavioral assessments of sheep were performed by recording time spent on food, rumination, idle, and other activities, through visual observation of the animals every 5 min, for 24 h [15]. The average number of chewing merícicas by ruminal boli, and the average time spent chewing merícica by ruminal boluses were obtained in three periods of 2 h, registering three values distributed in zones of 10–12 h, 14–16 h, and 18–20 h, by using a digital chronometer. For observations at night, the environment was maintained with artificial lighting. The results regarding factors of ingestive behavior were obtained using the following equations: FE = DMI/FT; RE = DMI/RT; ERndf = NDFI/RT; CTT = FT+ RT; BOL = RT/TCB; NCB = BOL/TCB, where FE (grams of DM h^{-1}) is feed efficiency, DMI (g day^{-1}) is dry matter intake, FT (h day^{-1}) corresponds to the time of feeding, RE (g DM h^{-1}; g NDF h^{-1}) is rumination efficiency, RT (h day^{-1}) is rumination time, CTT (h day^{-1}), total time spent chewing, BOL (No. day^{-1}) is the number of boli, TCB (s per bolus) is the time spent chewing per bolus (POLLI et al., 1996), NC (No day^{-1}) is the number of chews, and NCB (No dia^{-1}) is the number of chews per bolus. The boli was considered as the portion of food that returns to the mouth to undergo the process of rumination.

Data were initially subjected to normality (Cramer–von Mises) and homoscedasticity (Levene) tests. When the assumptions were met, analysis of variance was performed using F-test. In case of significant differences, means were compared by Student–Newman–Keuls (SNK) test at the 5 % probability level. Statistical analyses were run using the GLM procedure of SAS 9.0 software (2002).

For consumption data, digestibility and behavior were analyzed by using the following statistical model:

$$Y_{ijk} = \mu + H_j + G_k + HG_{jk} + e_{ijk}$$

where,

Y_{ijk} = Value for the observation of the repetition of the level of inclusion j versus chemical treatment k; μ = Overall average; H_j = Effect of level of inclusion j (j = 1, 2, 3, 4); G_k = Effect of chemical treatment k (k = 1, 2) HG_{jk} = Interaction of the effects of the inclusion level j to chemical treatment k; e_{ijk} = Random error associated with the observation.

For the data pertaining to pH, ammonia nitrogen in the rumen fluid and blood urea were analyzed by using the following statistical model:

$$Y_{ijklm} = \mu + H_i + G_j + (H \times G_ij) + \alpha_{ijk} + T_l + (H \times T_{il}) + (G \times T_{jl}) + (H \times G \times T_{ijl}) + \varepsilon_{ijklm},$$

where Y_{ijklm} This is the dependent variable; μ Overall average; H_i Effect of level of inclusion; Gj Effect of chemical treatment; (H x Gij) Interaction of the effects of the inclusion level to chemical treatment; α_{ijk} Effect of random error, where the variance of animals within the treatments (H + G + H x G); T_l It is the fixed effect of time of collection; (H x T) It is the fixed effect of the interaction between the level of inclusion and the time of collection; (G x T) It is the fixed effect of the interaction between the chemical treatment and the time of collection; (H x G x T) It is the fixed effect of the interaction between the levels of inclusion, chemical treatment and the time of collection; is ε_{ijklm} It is the effect of random error.

3. Results and Discussion

The interactions between levels of inclusion of the cashew byproduct and chemical treatments were not significant (P > 0.05) for dry matter intake (DMI) or intake of organic matter. No effect was observed (P > 0.05) on DMI by type of chemical treatment used in the cashew byproduct (Table 3).

The average daily consumption of dry matter intake was 1045, 1 g day^{-1}, greater than that observed by [16] when providing diets containing ammoniated elephant grass, cocoa meal, and palm kernel cake (928 g day^{-1}). [17] evaluated the inclusion of cane sugar *in natura* or ensiled with calcium oxide and urea in sheep diets and observed higher DMI in g day^{-1} for sugar cane diet treated with 0.5 % urea (748.86 g day^{-1}) than supplied *in natura* (618.07 g day^{-1}). The average consumption of experimental treatments was 683.47 g day^{-1}, which is lower than that found in the present study (1045.10 g day^{-1}).

No differences were observed (P > 0.05) in the consumption of organic matter between the diets. Consumption might not have differed between diets because the

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nutrient levels of the diets were not affected, making the diets isonutritive. Consumption was lower than that observed by [18] who also evaluated byproducts. Reductions in consumption might be associated with odors or unpleasant tastes and/or digestive effects on the rate of passage, which is due to the different profiles of each byproduct [19]. Animals might also adjust DMI and organic matter intake according to their energy intake [20].

The inclusion of different levels of cashew byproduct with chemical treatment (CT) and without chemical treatment (NCT) did not influence the consumption of protein. The average values of consumption were higher than those recommended by the [21]. Additional research is required to evaluate the form of chemical treatment required for the cashew byproducts and the consumption of metabolizable protein in diets that contain these byproducts should be measured to ascertain the most efficient use and absorption of dietary protein.

There was a significant interaction ($P > 0.05$) between levels of inclusion of cashew byproduct with CT and NCT byproduct on the consumption of ether extract (g day ^{1}) (Table 3). The consumption of ether extract in the diet containing 21 % of byproduct and NCT was higher (60.31 g day^{-1}) than the same level of inclusion of byproduct with CT (29.66 g day^{-1}). These data demonstrate that caution must be taken before adding chemically treated cashew byproduct at high levels of inclusion (above 11 %).

There was no ($P > 0.05$) interaction between the levels of inclusion of the cashew byproduct with CT or NCT on NDF intake (g day ^{1}). The average NDF intake of animals that received the cashew byproduct with CT was always higher than that of animals that received the cashew byproduct with NCT. There were differences between the levels of inclusion on the consumptions of NDF (g day ^{1}). According to [22], fiber can be defined as the structural components of plants (cell walls), the fraction of less digestible food, the fraction of the food that is not digested by enzymes in mammals, or the fraction of the food that promotes rumination and the health of the rumen.

The [21] does not specify values of minimum fiber consumption for sheep. However, Santa Inês sheep with an average live weight of 45 kg require at least 28.05 % NDF for adequate ruminal function [23]. In our study, all values exceeded this minimum recommendation.

There was no interaction ($P < 0.05$) between levels of inclusion of the cashew byproduct and CT or NCT of the byproduct on the coefficients of digestibility of DM, OM, and CP ($P > 0.05$) (Table 4). No differences were observed between the averages of inclusion and CT or NCT of the cashew byproduct for these parameters.

We observed interaction ($P < 0.05$) between levels of inclusion of cashew byproduct and CT or NCT of the byproduct (Table 4). A diet that included 21 % of the NCT cashew byproduct presented higher ether extract digestibility (86.82 %) than 21 % of the CT byproduct diet (67.73 %) ($P < 0.05$). The other levels of inclusion did not differ significantly ($P > 0.05$). This probably compromised the consumption of ether extract at this level for diets with CT, given the high correlation between digestibility and consumption of ether extract.

Consistent with our results, [16] also observed no statistical differences for the coefficients of digestibility of DM, OM, and CP when sheep were fed with diets containing ammoniated elephant grass, cocoa meal, and palm pie. The ammoniation did not interfere in the coefficient of digestibility of ether extract, differing from the results obtained in our study, but despite this, the average coefficient of digestibility observed here (81.76 %) was higher than that found by [16] (72.87 %). The average coefficient of digestibility of MS (58.83 %) was similar to the average values obtained by [24] (58.58 %) when sheep were provided with ammoniated rice straw. For the digestibility of the OM, values were similar to those reported by [16], being 65.07 % and 65.93 % respectively.

No interaction ($P > 0.05$) was observed between levels of inclusion of the cashew byproduct with CT or with NCT (Table 4). The material treated with urea showed higher digestibility of NDF, which can be coupled to the fact that the technique of ammoniation improved the quality of the fiber. [25] proposed that the effect of treatment with urea on digestibility of NDF was due to its effect of breaking the ester links between the components of the cell walls and the phenolic acids and the depolarization of lignin. Ammoniation promotes the increased fermentable carbohydrate content, which results in an increase in the digestibility and consumption of CT fibrous materials [6].

No interaction was observed ($P < 0.05$) between treatments and collection times (Table 5). Eight hours postprandial the pH levels of inclusion of 16 % and 21 % of the NCT cashew byproduct presented higher values that that of 16 % CT byproduct; these were similar to other levels of inclusion with or without chemical treatment. Collection of ruminal fluid 8 h postprandial presented an average pH of 8.01, higher than at other times of collection. The average pH values obtained in this study remained within the average recommended by [26], i.e., between 6.0 and 8.0, compatible with the action of the enzymes of these microorganisms. [27] pointed out that the pH is the factor with the most influence on the ruminal ecosystem. The bacteria that degrade cellulose and produce methane are quite sensitive to pH lower than 6.0.

The adjustment of the pH depends on the transit of

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fatty acids through the ruminal wall and the secretion of bases inside the wall [6]. According to this author, the urea can be rapidly hydrolyzed to ammonium bicarbonate.

We observed a significant interaction ($P < 0.05$) between the time of collection and level of inclusion of the byproduct and between the time of collection and level of inclusion and CT or NCT (Table 5). After 2 h, the postprandial levels of 11, 16, and 21 % CT cashew byproduct showed higher concentrations of NH_3-N in relation to the other collection times. The lowest concentration was observed in the level of 16 % NCT byproduct, similar to the levels of 6, 11, and 21 % NCT cashew byproduct.

These data revealed the possible imbalance in the dietary energy to protein ratio that elevated concentrations of ammoniacal nitrogen in the rumen fluid. [28] emphasized that it is of utmost importance to optimize ruminal microbial protein synthesis. For this to be possible, the most important factor, besides soluble nitrogen and a certain amount of pre-formed amino acids, is the available amount of fermentable organic matter for microorganisms, i.e., the availability of energy.

The lowest concentration of microbial protein was obtained when provided 16 % of NCT cashew byproduct. The highest concentration was obtained in the second hour postprandial hyperglycemia (15.39 mg dL^{-1}) and the lowest was for the time zero. A minimum concentration of 10 mg/100 ml of ammonia in the rumen is necessary to allow adequate microbial fermentation [6]. Moreover, [29], evaluating the effect of ammonia concentration on the production of microbial protein, concluded that 5 mg of ammonia per 100 mL of rumen contents is sufficient for the maintenance of the ruminal microorganisms.

There was no interaction ($P > 0.05$) between inclusion level, time of collection, and CT or NCT on the levels of plasma urea ($P > 0.05$). There were no significant differences between the times of collection. As for the levels of inclusion, considering the chemical treatment or not with urea, 6 % with CT had the highest concentration of serum urea. The 6 % level of inclusion of the cashew byproduct had the second largest concentration of NH_3-N in relation to the 21 % with CT level, with both being similar to other experimental treatments.

Ammonia is a primary derivative of amino acid catabolism by ruminal bacteria, removed by the liver, and incorporated into the urea cycle, resulting in the formation of urea and eventual excretion by kidneys [30]. The concentrations of serum urea can give evidence of ruminal protein availability and adequate supply of protein in the diet. Below normal values may indicate a deficiency in supply or pathological states; normal values of urea for sheep are between 18 and 31 mg dL^{-1} of serum.

There was no interaction ($P > 0.05$) between levels of inclusion and the chemical treatment for the time spent with food (TF), rumination (TR), idle, (TI) and other activities (TOA) (Table 6).

For the TF the 21 % level of inclusion provided greater time than the 11 and 16 % levels, these three were similar to the 6 % level. This increase in TF observed in the 21 % level of inclusion of byproduct could have been caused by the increase in the fiber content of the diet, as increasing the amount of fiber in diets stimulates the masticatory activity [31]. A fact evidenced by [32], who evaluated the effect of five levels of NDF (20, 27, 34, 41, and 48 %) in the diet of goats, and found an increase in the time of ingestion and rumination and reduction of idleness with the elevation of levels of NDF in the diet.

[32] described rumination as a physiological feature for the best utilization of food, which begins when the time of feed supply decreases. This was not observed in our study, perhaps because the efficiency of rumination (DM and NDF) (Table 7) was not affected by the levels of inclusion or by CT or NCT of the cashew byproduct. The TI and TOA also showed no differences between the levels of inclusion or between CT and NCT.

[32] evaluating the ingestive behavior of sheep fed diets composed of elephant grass silage ammoniated or not and agroindustrial byproducts, observed that the animals that consumed ammoniated elephant grass silage presented a shorter time spent with rumination.

[5] also observed differences in rumination time between experimental treatments, with a shorter rumination time at a greater level of inclusion of cashew byproduct (5.33) than the lower level of inclusion (4.19).

There was no interaction ($P < 0.05$) between levels of inclusion and chemical treatment on power efficiency (Table 7). The 16 % NCT level of inclusion (366.40) provided a better power efficiency than the same level of inclusion with CT (307.64) and was superior to other levels of inclusion with NCT. The diets containing 11 and 16 % of cashew byproduct promoted better efficiency of supply in relation to the levels of inclusion of 6 and 21 %. This probably occurred because these levels (11 and 16 %) presented shorter supply, since there was a high negative correlation between the time of food and energy supply ($r = -0.8512$; $P < 0.0001$).

There was no interaction ($P > 0.05$) between levels of inclusion and chemical treatment for the rumination efficiency parameters (DM and NDF), chewing time, total number of ruminal boli per day, the number of chews per day, the number of chews per boli, and chew times for boli (Table 7). The number of chews per day were higher in CT cashew byproduct (20.947) than in NTC byproduct

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(20.201). This may have occurred due to the higher value of NDF and ADF present in diets containing CT cashew byproducts.

4. Conclusions

Chemical treatment improved the digestibility of fiber, consequently improved the consumption of fibrous constituents, and did not alter the intake of dry matter, organic matter, or crude protein.

The levels of inclusion of byproduct did not affect ingestive behavior, feed efficiency, or efficiency of rumination. The chemical treatment increased the time spent chewing.

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Appendixes: Tables

Table 1. Chemical composition of ingredients menus offered to sheep

Component	*By-product of cashew without chemical treatment*	*By-product of cashew with chemical treatment*	*Hay of Aruana*	*Grain of corn*	*Cotton cake*
Dry matter[a]	88.32	90.52	87.26	88.39	92.70
Crude protein[b]	15.28	23.88	9.09	11.24	27.63
NDIN[b]	2.36	3.60	1.33	1.65	1.27
ADIN[b]	2.30	3.74	1.27	0.08	1.16
Ether extract[b]	4.00	0.76	2.94	3.85	9.24
NDF[b]	68.34	86.97	85.26	31.68	55.93
ADF[b]	47.89	62.45	45.73	3.90	38.94
Hemicelluloses[b]	20.45	24.52	39.54	27.77	16.99
Cellulose[b]	22.70	6.49	38.81	3.73	28.32
Lignin[b]	21.84	62.08	4.22	0.31	10.58
Ash[b]	5.18	4.25	8.73	1.60	4.34
Ca[b]	0.80	0.77	1.19	0.88	1.09
TC[b]	75.54	71.11	79.24	83.31	58.79
NFC[b]	21.58	7.69	1.92	52.13	10.11
TDN[b]	44.67	8.65	55.44	77.22	71.06

Note: [a]% natural matter; [b]% the dry matter; DM=Dry matter; CP=Crude protein; NDIN = Neutral detergent insoluble nitrogen; ADIN = acid detergent insoluble nitrogen; NDF = neutral detergent fiber; ADF = acid detergent fiber; CT = Total Carbohydrates; NFC = non-fibrous carbohydrates; NDT = total digestible nutrients as NRC (2001)

Table 2. The chemical composition of the experimental diets

Componentes	**Experimental diets**							
	6		11		16		21	
	Without chemical treatment	With chemical treatment	Without chemical treatment	With chemical treatment	Without chemical treatment	With chemical treatment	Without chemical treatment	With chemical treatment
Dry matter[a]	89.42	89.54	88.80	89.04	88.98	89.33	89.35	89.81
Crude protein[b]	15.86	16.35	13.87	14.84	14.68	16.03	14.37	16.18
NDIN[b]	1.50	1.57	1.58	1.72	1.61	1.80	1.67	1.93
ADIN[b]	0.82	0.90	0.84	1.01	0.94	1.17	1.02	1.32
Ether extract[b]	5.25	5.07	4.52	4.16	4.74	4.23	4.58	3.90
NDF[b]	54.10	55.17	54.85	56.95	55.59	58.53	56.55	60.48
ADF[b]	27.10	27.93	26.44	28.09	28.26	30.55	29.11	32.19
Hemicelluloses[b]	27.00	27.23	28.40	28.86	27.33	27.97	27.44	28.29
Cellulose[b]	20.73	19.81	19.80	17.97	20.36	17.80	20.30	16.88
Lignin[b]	5.57	7.86	5.57	10.11	6.75	13.09	7.51	16.00

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Ash[b]	4.36	4.31	4.45	4.35	4.48	4.33	4.54	4.34
Ca[b]	1.01	1.01	0.99	0.99	0.99	0.98	0.98	0.97
TC[b]	74.55	74.29	77.16	76.66	76.11	75.41	77.11	76.18
NFC[b]	25.55	24.75	27.59	26.00	26.40	24.19	26.31	23.34
TDN[b]	65.79	78.08	67.35	60.07	57.41	65.81	64.64	62.39

Note: [a]% natural matter; [b]% the dry matter; DM=Dry matter; CP=Crude protein; NDIN = Neutral detergent insoluble nitrogen; ADIN = acid detergent insoluble nitrogen; NDF = neutral detergent fiber; ADF = acid detergent fiber; CT = Total Carbohydrates; NFC = non-fibrous carbohydrates; NDT = total digestible nutrients as NRC (2001)

Table 3. Daily intake of dry material and nutrient requirements of sheep fed levels of inclusion of the byproduct of cashew with or without chemical treatment

Chemical treatment	Levels of inclusion of the byproduct of cashew (%)				Means
	6	11	16	21	
Dry matter intake (g day^{-1}) (CV=17.88%)					
Without chemical treatment	921.93	1001.41	1090.28	1093.43	1026.76^{A}
With chemical treatment	1011.79	1184.21	1068.10	989.66	1063.44^{A}
Means	966.86^{a}	1092.81^{a}	1079.19^{a}	1041.55^{a}	
Organic matter intake (g day^{-1}) (CV=17.92%)					
Without chemical treatment	894.80	972.56	1057.80	1059.28	996.11^{A}
With chemical treatment	976.00	1143.45	1029.30	952.06	1025.20^{A}
Means	935.40^{a}	1058.01^{a}	1043.01^{a}	1005.67^{a}	
Crude protein intake (g day^{-1}) (CV=16.80%)					
Without chemical treatment	158.03	169.84	181.55	188.45	174.47^{A}
With chemical treatment	197.97	191.26	168.14	149.03	176.60^{A}
Means	177.10^{a}	170.55^{a}	174.81^{a}	168.74^{a}	
Ether extract intake (g day^{-1}) (CV=16.24%)					
Without chemical treatment	50.77	54.17	58.30	60.31	55.89^{A}
With chemical treatment	54.91	52.87	39.34	29.66	44.19^{B}
Means	52.84^{a}	53.52^{a}	48.82^{a}	44.99^{a}	
Neutral detergent fiber intake (g day^{-1}) (CV=18.90%)					
Without chemical treatment	477.89	520.77	567.52	546.74	528.23^{B}
With chemical treatment	564.76	686.11	652.22	658.61	640.43^{A}
Means	521.33^{a}	603.44^{a}	609.87^{a}	602.67^{a}	

Note: Means followed by different letters uppercase and lowercase letters columns in the lines differ by SNK test at 5% probability.

Table 4. Coefficients of digestibility of dry material and nutrients in sheep fed with levels of inclusion of the byproduct of cashew with or without chemical treatment

Chemical treatment	Levels of inclusion of the byproduct of cashew (%)				Means
	6	11	16	21	
Digestibility of dry matter (%) (CV=10.14%)					
Without chemical treatment	59.22	62.69	52.77	59.99	58.67^{A}
With chemical treatment	60.62	54.90	62.24	58.17	58.98^{A}
Means	59.92^{a}	58.80^{a}	57.50^{a}	59.09^{a}	
Digestibility of organic matter (%) (CV=8.17%)					
Without chemical treatment	66.53	68.53	59.17	65.36	65.23^{A}
With chemical treatment	66.49	62.04	67.49	64.92	64.90^{A}
Means	66.51^{a}	65.28^{a}	63.33^{a}	65.14^{a}	

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Digestibility of crude protein (%) (CV=11.76%)					
Without chemical treatment	60.72	56.71	49.27	56.49	55.80^{A}
With chemical treatment	68.57	50.63	60.54	59.02	59.69^{A}
Means	64.65^{a}	53.67^{a}	54.91^{a}	57.76^{a}	
Digestibility of ether extract (%) (CV=6.54%)					
Without chemical treatment	82.83^{Aa}	86.82^{Aa}	82.54^{Aa}	86.82^{Aa}	85.35
With chemical treatment	84.15^{Aa}	83.37^{Aa}	77.38^{Aa}	67.73^{Bb}	78.16
Means	83.49	85.09	79.96	78.47	
Digestibility of neutral detergent fiber (CV=20.51%)					
Without chemical treatment	46.49	47.79	31.57	35.63	40.37^{B}
With chemical treatment	50.36	43.97	53.60	50.60	49.39^{A}
Means	48.42^{a}	42.38^{a}	42.59^{a}	43.12^{a}	

Note: Means followed by different letters uppercase and lowercase letters columns in the lines differ by SNK test at 5% probability.

Table 5. Average values of pH, ammoniacal nitrogen (NH_3-N) in the ruminal fluid and blood urea (mg dL^{-1}) of sheep fed levels of inclusion of the byproduct of cashew with or without chemical treatment

Levels of inclusion (%)	Treatment	Collection times (Hours)				
		0	2	5	8	Means
pH						
6	Without chemical treatment	7.53^{Aa}	7.13^{Aa}	7.50^{Aa}	7.93^{Aa}	7.53^{AB}
	With chemical treatment	7.47^{Aa}	7.17^{Aa}	7.53^{Aa}	8.60^{Aa}	7.69^{AB}
11	Without chemical treatment	7.83^{Aa}	7.53^{Aa}	6.53^{Aa}	7.40^{ABa}	7.33^{AB}
	With chemical treatment	7.50^{Aa}	7.30^{Aa}	7.70^{Aa}	8.07^{ABa}	7.64^{AB}
16	Without chemical treatment	7.83^{Aa}	7.53^{Aa}	8.23^{Aa}	8.70^{Aa}	8.08^{A}
	With chemical treatment	6.60^{Aa}	7.97^{Aa}	7.47^{Aa}	6.73^{Ba}	7.04^{B}
21	Without chemical treatment	7.80^{Aa}	7.33^{Aa}	8.03^{Aa}	8.40^{Aa}	7.09^{A}
	With chemical treatment	7.70^{Aa}	7.00^{Aa}	7.40^{Aa}	8.27^{ABa}	7.59^{AB}
Means		7.53^{b}	7.30^{b}	7.55^{b}	8.01^{a}	
NH_3-N (mg dL^{-1})						
6	Without chemical treatment	2.99^{Ab}	11.95^{BCa}	8.46^{Ba}	10.21^{Ba}	8.40^{CD}
	With chemical treatment	5.27^{Ab}	14.49^{Ba}	16.03^{Aa}	12.01^{ABa}	11.95^{AB}
11	Without chemical treatment	4.74^{Ab}	11.98^{BCa}	11.23^{ABa}	9.97^{Ba}	9.48^{BCD}
	With chemical treatment	3.48^{Ac}	19.86^{Aa}	12.64^{ABb}	15.90^{Aab}	12.97^{A}
16	Without chemical treatment	5.77^{Aa}	8.02^{Ca}	8.00^{Ba}	6.01^{Ba}	6.95^{D}
	With chemical treatment	3.21^{Ab}	24.96^{Aa}	8.16^{Bb}	5.42^{Bb}	10.44^{ABC}
21	Without chemical treatment	4.89^{Ab}	11.02^{BCa}	11.54^{ABa}	8.97^{Bab}	9.11^{BCD}
	With chemical treatment	2.76^{Ac}	20.79^{Aa}	9.77^{Bb}	6.01^{Bbc}	9.83^{BCD}
Means		4.14^{c}	15.39^{a}	10.73^{b}	9.31^{b}	
Urea plasm (mg dL^{-1})						
6	Without chemical treatment	38.82^{Aa}	39.25^{Aa}	26.48^{Ba}	38.42^{Aa}	35.74^{B}
	With chemical treatment	39.83^{Aa}	48.63^{Aa}	49.66^{Aa}	34.97^{Aa}	43.27^{A}
11	Without chemical treatment	28.99^{Aa}	27.41^{Aa}	32.39^{Aba}	37.31^{Aa}	31.53^{BC}
	With chemical treatment	22.35^{Aa}	29.62^{Aa}	24.72^{Ba}	31.46^{ABa}	27.04^{BC}
16	Without chemical treatment	28.94^{Aa}	33.74^{Aa}	27.08^{Ba}	36.89^{Aa}	31.66^{BC}
	With chemical treatment	23.01^{Aa}	34.17^{Aa}	33.62^{Ba}	33.84^{Aa}	31.16^{BC}

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21	Without chemical treatment	32.74^{Aa}	30.17^{Aa}	27.83^{Ba}	18.13^{ABa}	27.22^{BC}
	With chemical treatment	16.55^{Aab}	33.54^{Aa}	30.45^{ABab}	13.73^{Bb}	23.57^{C}
Means		28.90^{a}	34.57^{a}	31.53^{a}	30.59^{a}	

Note: [1] Letters equal in the same column indicate statistical similarity to 5% (SNK) [2] Lowercase equal in the same line indicate statistical similarity to 5% (SNK) CV= 23.06%

Table 6. Feeding behavior of sheep receiving different levels of inclusion of the byproduct of cashew with or without chemical treatment

Chemical treatment	**Levels of inclusion of the byproduct of cashew (%)**				**Means**
	6	11	16	21	
Feeding Time (CV=16.95%)					
Without chemical treatment	4.56	4.08	3.06	5.61	4.33^{A}
With chemical treatment	5.14	3.94	4.64	4.94	4.67^{A}
Means	4.85^{ab}	4.01^{b}	3.85^{b}	5.28^{a}	
Ruminantion time (CV=22.44%)					
Without chemical treatment	6.08	6.67	6.47	5.78	6.25^{A}
With chemical treatment	5.69	6.67	7.19	7.00	6.64^{A}
Means	5.89^{a}	6.67^{a}	6.83^{a}	6.39^{a}	
Idle time (CV=18.66%)					
Without chemical treatment	7.64	9.86	8.64	7.06	8.30^{A}
With chemical treatment	6.97	7.06	7.97	6.44	7.11^{A}
Means	7.31^{a}	8.46^{a}	8.31^{a}	6.75^{a}	
Others activities time (CV=21.62%)					
Without chemical treatment	5.72	3.39	5.83	5.56	5.13^{A}
With chemical treatment	6.19	6.33	4.19	5.61	5.58^{A}
Means	5.96^{a}	4.86^{a}	5.01^{a}	5.58^{a}	

Note: Means followed by different letters uppercase and lowercase letters columns in the lines differ by SNK test at 5% probability.

Table 7. Food efficiency and nictemeral pattern of sheep receiving different levels of inclusion of the byproduct of cashew with or without chemical treatment

Chemical treatment	**Levels of inclusion of the byproduct of cashew (%)**				**Means**
	6	11	16	21	
Feeding efficiency (CV=21.50%)					
Without chemical treatment	213.14^{Ab}	245.18^{Ab}	366.40^{Aa}	201.23^{Ab}	256.49
With chemical treatment	197.56^{Aa}	307.64^{Aa}	230.57^{Ba}	202.50^{Aa}	234.57
Means	205.35	276.41	298.49	201.86	
Rumination efficiency (DM) (CV=20.03%					
Without chemical treatment	152.04	154.79	168.01	194.62	167.37^{A}
With chemical treatment	177.62	185.01	156.61	141.01	165.06^{A}
Means	164.83^{a}	169.90^{a}	162.31^{a}	167.81^{a}	
Ruminantio efficiency (NDF) (CV=20.21%)					
Without chemical treatment	78.98	79.84	87.39	97.53	85.93^{A}
With chemical treatment	99.17	107.23	95.81	93.52	98.93^{A}
Means	89.08^{a}	93.53^{a}	91.60^{a}	95.53^{a}	
Chews total time (CV=12.98%)					
Without chemical treatment	10.64	10.83	10.75	9.53	10.58^{A}

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With chemical treatment	10.83	10.61	11.83	11.94	11.31^{A}
Means	10.74^{a}	10.68^{a}	10.68^{a}	11.67^{a}	
Number of boli daily ruminantion (CV=26.49%)					
Without chemical treatment	554.77	579.62	590.06	393.22	529.42^{A}
With chemical treatment	560.57	580.11	614.05	210.59	491.33^{A}
Means	557.67^{a}	579.87^{a}	602.05^{a}	301.90^{b}	
Number of chews daily (CV=26.79%)					
Without chemical treatment	21.884	24.093	23.465	11.364	20.201^{B}
With chemical treatment	20.298	22.975	26.561	13.956	20.947^{A}
Means	21.091^{c}	23.534^{b}	25.013^{a}	12.660^{d}	
Number of chews boli (CV=17.84%)					
Without chemical treatment	57.93	62.59	58.96	57.78	59.32^{A}
With chemical treatment	61.37	64.48	61.74	66.03	63.41^{A}
Means	59.65^{a}	63.54^{a}	60.35^{a}	61.91^{a}	
Chewings time for boli (CV=14.08%)					
Without chemical treatment	41.78	42.63	40.37	41.15	41.48^{A}
With chemical treatment	42.48	42.44	40.04	41.44	41.60^{A}
Means	42.13^{a}	42.54^{a}	40.23^{a}	41.30^{a}	

Note: Means followed by different letters uppercase and lowercase letters columns in the lines differ by SNK test at 5% probability.

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ARTICLE

A Simple Procedure for Extraction of Surface Protein of *Salmonella Serotypes* and *Escherichia coli* Strains Isolated from Poultry and Pigs

Tran Thi Quynh Lan* **Doan Thi Da Linh** **Le Ho Truc Phuong**

Department of Veterinary Biosciences, Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh City, Vietnam

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ABSTRACT

Salmonella and *E.coli* possess different surface protein structures that can induce protective immune responses. Identification of these proteins capacitates development of diverse applications in prevention and diagnosis that contribute to effectively control disease-causing enterobacteria pathogens such as *Salmonella* and *E.coli*. A simple procedure for obtaining protein complexes of *Salmonella* serotypes and *E.coli* is performed in this study. A sonication process with heat treatment of whole bacteria induced the release of protein complexes. Concentration of the protein extract was quantified using protein quantification Kits-Rapid, and protein complex profile was obtained by SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis) and silver staining. The concentrations of protein ranged from 29.45 to 45.35 µg/mL in the *Salmonella* protein extracts, and from 25.35 to 36.72 µg/mL in the *E.coli* protein extracts. Six major groups of proteins from *E. coli* (YfiO, NipB, OmpF, YfgL, Talc, YaeT) and four major groups of proteins from *Salmonella* (Flagellin, OmpA, Porin, SEF21) were preliminarily determined by a simple procedure of extraction based on the molecular weight.

1. Introduction

Salmonella and *E.coli* are common bacteria in intestinal tract that is responsible for a variety of intestinal disorders in swine and poultry [1]. The expression of *E.coli* and *Salmonella* virulence genes causes gastrointestinal diseases in animals as well as intoxications in humans using the meat contamination bacteria [2]. In order to reduce the intensive intestinal colonisation of pathogenic *Salmonella* and *E.coli* in commercial farms, various strategies have been applied, such as antibiotics supplementation in the diet or supplementation of additives that inhibit bacterial adhesion to the intestinal epithelium, competitive exclusion by non-pathogenic bacteria, genetic selection of animal strains, and development of vaccines [3].

Enterobacteriaceae possesses different surface structures (proteins or antigens) that can induce protective immune responses [4]. The utilization of an optimized method for surface protein extraction should greatly enable applications for the therapy and diagnosis including the production of ELISA kit and subunit vaccine as well as other proteomic research. Nowadays, the protein extraction from the bacteria surface was done by many

**Corresponding Author:*
Tran Thi Quynh Lan,
Department of Veterinary Biosciences,Faculty of Animal Science and Veterinary Medicine, Nong Lam University, Ho Chi Minh City, Vietnam;
Email: lan.tranthiquynh@hcmuaf.edu.vn

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methods such as the cell surface protein isolation kit, the use of a rotating cylinder probe [5] or a combination of detergent and phase separation using Triton X-114 (TX-114) [6]. However, most of these methods have limitations due to augmentation of performance duration, the expense as well as special materials in laboratory.

The aim of this study is to determine a simple extraction procedure for obtaining the surface protein complexes of *Salmonella* serotypes and *E.coli* strains. This is the first step to identify immune-reactive proteins in the next approach. Therefore, identification of protein components obtaining from extraction was carried out by examining the molecular weight of proteins separated in SDS-PAGE bands profile.

2. Materials and Methods

2.1 Bacterial Strains

The selection of bacterial strains was the first step in the protein extraction process. A total of 17 *Salmonella* serotypes and *E.coli* strains isolated from poultry and pigs from previous study were carried out in this study (Table 1). *Salmonella* Typhimurium ATCC® 14028™ was used as control. All of bacterial strains were stored at -70°C in glycerol 25 % [7].

Table 1. *Salmonella* serotypes and *E.coli* strains

Bacterial strains	Fecal samples	Quantity	Identification code
Salmonella serotypes*			
*S.*Typhimurium 14028™	control	1	ATCC
S. Typhimurium	pig	2	ST1, S5
S. Paratyphi A	pig	1	ST5
S. Senftenberg	pig	1	ST7
S. Saintpaul	pig	1	SR6
S. Montevideo	pig	1	S6
S. Anatum	pig	1	S7
S. Paratyphi B	pig	1	S8
S. Enteritidis	chicken	2	S9, S10
E.coli strains **	chicken	1	E3
	chicken	1	E4
	chicken	1	E5
	chicken	1	E6
	chicken	1	E7
	chicken	1	E8

Note: (*) Provided by Laboratory of Department of Veterinary Biosciences, FMVS, NLU-HCMC [7, 8] , and (**) Laboratory of Department of Veterinary Public Health, FMVS, NLU-HCMC

2.2 Antigenic (Protein) Extract Complexes of Bacterial Strains

The production of surface protein complexes was carried out from the whole bacterial cells. Briefly, a colony isolated on blood agar (Nam Khoa Co.Ltd, HCMC,VN) was incubated in 100 mL Brain Heart Infusion media (BHI) (HiMEdia Laboratories Pvt. Ltd) (pH 7.4) overnight at 37°C with shaking of 150 rpm (until OD_{600} = 0.65) in shaker inoculator machine (Hanbaek Scientificco, HB-201SF, Korea). The bacterial suspension was centrifuged at 2400 x g for 20 minutes at room temperature by centrifuge machine (Hanil Science Industrial, FLETA 5, Korea) and then the pellet was suspended in 5 mL of Phosphate Buffer Saline (PBS) (pH 7.4). The suspension of bacterial cells was sonicated 20 times x 10 seconds on ice (Hwashin technology, powersonic 410, Korea), and then was centrifuged (11000g; 10 min.; 4°C) by cool centrifuge machine (Hanil Science Industrial, centrifuge Smart-R17, Korea). The supernatant was dialyzed and stored at -70°C.

Protein concentrations were determined by Quantification Kit-Rapid (51254, Sigma-Aldrich) (High Sensitivity Assay for Microplate reader) with bovine serum albumin (BSA) as standard.

2.3 SDS-PAGE Procedure

Briefly, samples (25-75 µg of total protein extract) were resolved by one-dimensional sulphate polyacrylamide gel electrophoresis (SDS-PAGE, Amersham, Buckinghamshire, UK). Protein samples were solubilised in buffer (0.5M Tris, pH 6.8, 2% sodium dodecyl sulphate (SDS), 10% glycerol, 5% mercaptoethanol) at 100°C for 5 min, and then loaded into 10% Bis-Acrylamide (Fisher scientific, FairLawn, New jersey, USA) separating gels. The gels were run at room temperature in buffer (25mM Tris, 0.2M glycine, 0.1% SDS) at 40V/3h and then 50V for sufficient time when the dye front reached the bottom of the gel casing. They were stained with silver and the apparent molecular masses of the proteins in the antigenic extracts were determined by PD Quest software (Bio-Rad) and then, comparing their electrophoretic mobility with that of the molecular mass markers (Precision Plus ProteinTM Standards, Bio-Rad).

Results of the procedure and protein identification of bacterial strains were demonstrated in OD_{595} values, protein concentrations (µg/mL of complex extract) and SDS-PAGE bands profiles. In term of procedure parameters, the main points focused on optic density of bacteria culture and sonication of bacteria suspension.

3. Results and Discussion

3.1 Procedure Parameters

Optic density (OD): Optic density of bacteria culture was measured at achieve the stationary phase in standard curves of bacteria growth. At that phase, the number of bacteria was high and stable so the concentration of bacterial protein would be at higher quantity.

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According to the previous procedure of growth conditions [9], the OD_{600} results of bacterial culture suspensions is shown in Table 2.

Table 2. OD_{600} of *Salmonella* serotype and *E.coli* strains

Bacterial identification code	OD_{600} average
ATCC	0.99
ST1	0.97
S5	1.06
ST5	1.06
ST7	1.03
SR6	1.01
S6	1.05
S7	1.12
S8	1.10
S9	1.00
S10	0.85
E3	0.96
E4	1.00
E5	0.95
E6	0.96
E7	1.03
E8	0.86

OD_{600} of *Salmonella* culture suspensions ranged from 0.85 to 1.12 that is higher than the standard curve, which is due to increasing of the growth of bacteria strains. That means the bacterial strains in the study possessed possibly high virulence. *Salmonella* strains isolates from pigs increased the growth in comparision to *Salmonella* Enteritidis isolates from chickens. Among all *Salmonella* isolates used in this study, the predominant genotypic virulence profile (virulotype) was characterized by the concomitant presence of *invA*, *sopB*, and *stn* in carrier strains. In contrast, two virulotypes comprising either *invA*, *sopB*, *spvC*, and *stn* or *invA* and *sopB* were identified for the *Salmonella* Typhimurium isolates. Virulotypes made up of multiple virulence genes were predominant in most *Salmonella* strains tested in the previous study, indicating that pigs might act as a reservoir for these virulent strains [7]. In contrast, the optic density was ranged at lower level from 0.86 to 1.03 from *E.coli* culture suspensions. All *E.coli* strains were isolated from clinically sick chickens; however, the virulence of these strains was still not investigated in our previous study.

Sonication: The bacteria suspension was destroyed by sonication wave at high temperature and then immediately was quickly chilled by ice at 4^0C. The sudden change in temperature causes to destroy bacterial cell wall. In our study, times of sonication were increased to 20 times at the highest frequency (40 kHz) to ensure that bacterial whole cell was completely broken.

3.2 Protein Concentration in Complexes Extract

Table 3. Quantification of *Salmonella* protein in extract

Salmonella serotypes	Bacterial identification	OD_{595}	Protein concentration (µg/mL)
Salmonella Typhimurium	ST1	0.97	37.3
	S5	1.06	41.05
Salmonella Paratyphi A	ST5	1.06	29.45
Salmonella Senftenberg	ST7	1.03	36.6
Salmonella Saintpaul	SR6	1.01	31.75
Salmonella Montevideo	S6	1.05	45.35
Salmonella Anatum	S7	1.12	35.3
Salmonella Paratyphi B	S8	1.10	33.95
Salmonella Enteritidis	S9	1.00	37.85
	S10	0.85	41.25
Salmonella Typhimurium	ATCC	1.27	76.4

Table 4. Quantification of *E.coli* protein in extract

Sample	OD_{595}	Protein concentration (µg/mL)
E3	0.96	25.35
E4	1.00	25.5
E5	0.95	32.55
E6	0.96	27.33
E7	1.02	36.72
E8	0.86	32.79

Concentrations of proteins was ranged from 29.45 µg/mL (*S.* Paratyphi A) to 45.35 µg/mL (*S.* Montevideo) in *Salmonella* serotypes (Table 3), and between 25.35-36.72 (µg/mL) in *E.coli* extracts (Table 4). The highest concentrations were approximately equivalent in *S.* Typhimurium and *S.* Enteritidis although the two serotypes were isolated from different hosts. The protein concentrations of extracts depend on many factors. The most important is the manifestation of the virulence factor, which is strains specificity. These two serotypes are dominant serotypes in many countries and also the most important cause of foodborne intoxication throughout the world, so that is a major challenge to the industry. The second factor is the quality of the extraction process in our experience. Protein concentrations of extracts were relatively linear in all bacterial samples and sufficient to use SDS-PAGE method for characterization of protein complexes.

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3.3 Identification of Protein Complexes

The SDS-PAGE profile of protein extract obtained from five *Salmonella* serotypes (*S.* Montevideo, S6; *S.* Paratyphi B, S8; *S.* Senftenberg, ST7; *S.* Enteritidis, S10 and *Salmonella* Typhimurium ATCC® 14028™, *Salmonella* enterica subsp.enterica) and three *E.coli* strains (E_5, E_7, E_8).

For *Salmonella* serotypes analysis (Figure 1), the frequency of the bands mostly appeared from *Salmonella* Typhimurium ATCC (96%) and *S.* Senftenberg, ST7 (85%) samples. The dominant protein obtained from protein extract of *Salmonella* serotypes is indicated with apparent molecular weights of 53 kDa (flagellin). This antigen, easily exposed to antibodies, has variability (it belongs to *Salmonella* serotyping proteins) and a time-regulated expression (phase depending) that represent some drawbacks, considering its use as a vaccine antigen [9]. There are other major proteins in the extracst that appear by SDS-PAGE with apparent molecular weights of 45, 35 , 34, 28.4, 26.8, 25.2, 23.2, 22.1, and 21 kDa (SEF21). These results were also described by Ochoa-Repáraz [10]. In fact, different authors have taken advantage of this property of these components for the development of specific serodiagnostic tests, like flagellin in ELISA.

For *E.coli* strains analysis (Figure 2), major proteins in the protein complexes extract appeared with apparent molecular weights of 26kDa (YfiO), 34kDa (NipB), 37kDa (OmpF), 40kDa (YfgL), 50kDa (TolC) and 85kDa (YaeT). The results may correspond with some of the major antigenic proteins from protein complexes of the *E.coli* cell envelope described by Stenberg (2005) [11] and Lasserre (2006) [12].

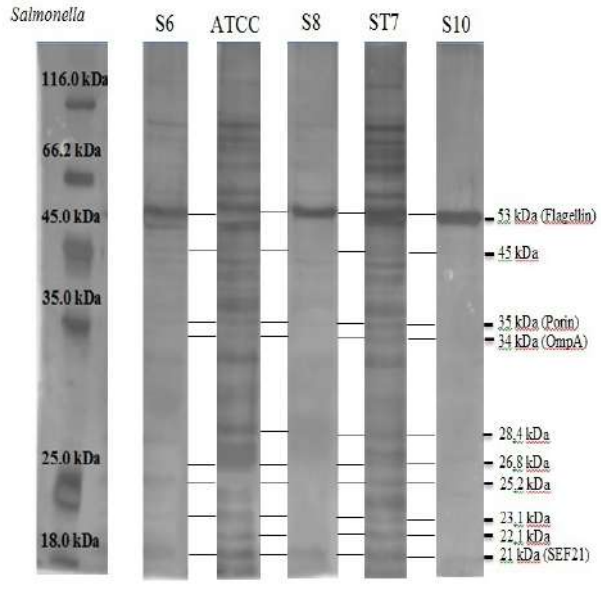

Figure 1. SDS PAGE of protein complexes extract of *Salmonella* serotypes (silver staining), and the position of some identify bands (arrows)

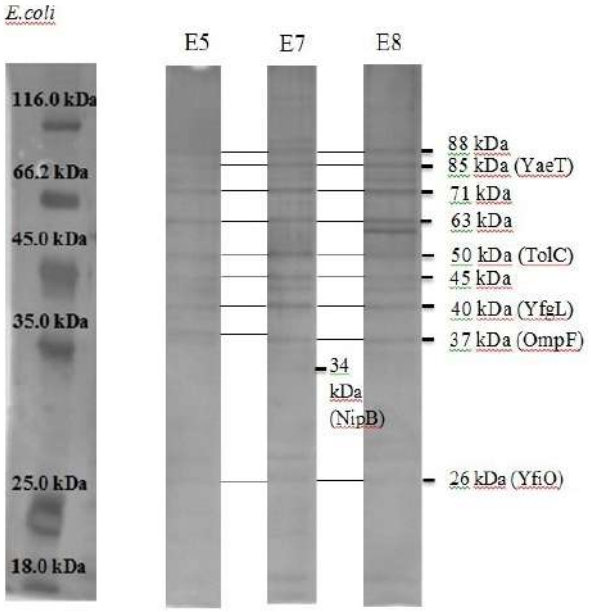

Figure 2. SDS PAGE of protein complexes extract of *E.coli* strains (silver staining), and the position of some identify bands (arrows)

4. Conclusions

Salmonella serotypes and *E.coli* strains possess antigenic protein structures that can induce protection in poultry and pigs. We obtained an antigenic protein complexes of *Salmonella* and *E.coli* from whole bacteria cells by a simple procedure. Further evaluation will be necessary to assess the immunogenicity of these antigens.

Acknowledgments

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ARTICLE

Multiple RT-PCR Detection of H5, H7, and H9 Subtype Avian Influenza Viruses and Newcastle Disease Virus

Feng Fei*

Jiaxing Vocational & Technical College, Jiaxing, Zhejiang, 314036, China

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ABSTRACT

Objective: This paper focuses on the multiple detection RT-PCR technology of H5, H7, AND H9 subtype avian influenza viruses and Newcastle disease virus, and points out the specific detection methods and detection procedures of avian influenza and Newcastle disease virus. **Methods:** The genes of Newcastle disease virus carrying out the HA gene sequence of H5, H7 and H9 subtype AIV in GenBank were used to establish a strategy for simultaneous detection of three subtypes of avian influenza virus and Newcastle disease virus. **Results:** The results showed that the program can detect and distinguish H5, H7 and H9 subtype avian influenza viruses and Newcastle disease virus at one time. **Conclusion:** Multiple RT-PCR detection method has high detection sensitivity and can detect and determine different subtypes of avian influenza virus and Newcastle disease virus quickly and accurately, therefore, it has a crucial role in the detection and control of avian influenza H5, H7 and H9 subtypes and Newcastle disease.

1. Introduction

AIV is a poultry strong infectious disease caused by the Orthomyxoviridae influenza Type A virus. The susceptible animals mainly include poultry and wild birds such as seabirds, waterfowl and wild birds. It also poses a serious threat to the health of humans and lower mammals, causing a variety of serious diseases. The spread of AIV can cause respiratory infections in poultry and in large cases cause large-scale deaths of poultry, causing serious economic losses to aquaculture enterprises. Avian influenza virus strains have large variations and many subtypes, which are characterized by rapid spread and difficult to control. As an internationally recognized Class A infectious disease, the avian flu virus will cause serious damage to social development and people's health, therefore, it is necessary to strengthen the research on avian influenza virus, and to identify various subtypes of avian influenza virus through scientific and reasonable detection methods, and provide data support for the prevention and treatment of avian influenza diseases.

Both Newcastle disease virus and avian influenza virus are infectious diseases with poultry as the main carrier. They are all respiratory diseases. The avian influenza virus and Newcastle disease virus have a high mortality rate and strong contagiousness, once poultry infects these two viruses; it is prone to widespread spread and brings very serious economic losses to the aquaculture enterprises. Diseased poultry infected with Newcastle disease virus or avian influenza virus manifests as neurological symptoms and respiratory symptoms. It is difficult to determine the relevant symptoms and the cause of the disease in time

**Corresponding Author:*
Feng Fei,
Jiaxing Vocational & Technical College, No. 1123 Changsheng South Road, Jiaxing, Zhejiang, 314036, China;
E-mail: 1328449833@qq.com.

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for anatomical examination, which brings certain difficulties to the prevention and treatment work. Conventional epidemic detection methods mainly include serological laboratory tests and pathogen isolation and identification. These methods are relatively complicated in operation and cumbersome in steps, and it is difficult to simultaneously diagnose and diagnose multiple pathogens.

2. Overview of Multiple RT-PCR

Multiple RT-PCR generally refers to an experimental method of amplifying multiple templates by using multiple pairs of primers in one PCR reaction system or adding multiple pairs of primers to one PCR reaction system to amplify several regions of a single template. Multiple RT-PCR technology can realize the effect of one PCR reaction monitoring multiple viral RNAs, which is indispensable in clinical virus monitoring and infection source determination process. Especially in the field of clinical infection differential diagnosis and import and export animal quarantine and immunization, there are very wide applications. Many scholars at home and abroad have carried out a lot of research on multiple RT-PCR technology. For example, Chinese scholars have established double PCR technology to detect IBV, NDV, double PCR detection of MG and MS, and multiple PCR technology to detect IBV, NDV, MG, ILTV, etc[1]. Zou et al. used RT-PCR technology in the 1990s in addition to the A-type avian influenza virus, the B-type avian influenza virus, and the C-type avian influenza virus[2]. In the 21st century, Ming et al. identified 15 avian influenza subtypes using RT-PCR technology[3].

The application of multiple RT-PCR detection method for the clinical diagnosis of diseases can significantly speed up the detection speed and detection accuracy, reduce the detection cost, and provide sufficient data support for the prevention and treatment of related infectious diseases.

This experiment uses multiple RT-PCR detection technology to establish a rapid detection method for H5, H7 and H9 subtype avian influenza viruses and Newcastle disease virus, which can timely obtain the types of related diseases and identify suspected cases of AIV and NDV infection, which provides theoretical guidance for the clinical diagnosis of avian influenza H5, H7 and H9 subtypes and Newcastle disease.

3. Materials and Methods

3.1 Materials and Reagents

AIV-H5 strain, AIV-H7 strain, AIV-H9 strain, and chicken Newcastle disease virus were purchased by Harbin Veterinary Research Institute. RT-PCR kit, RNA extraction kit, gel recovery kit, reverse transcriptase AMV, reaction buffer, and reverse transcription random primers were all provided by Nanjing Kingsray Biotechnology Co., Ltd.

3.2 Primer

Using DNAstar software, multiple pairs of primer designs were performed according to the H5, H7, AND H9 subtype avian influenza virus genes and Newcastle disease virus gene sequences registered in GenBank. After repeated experiments, a number of pairs of specific primers, H5 subtype avian influenza primers P1 and P2, were selected and the fragment length was 427 bp; H7 subtype avian influenza primer P3, P4, fragment length 501 bp; H9 subtype avian influenza primer P5, P6, fragment length 303 bp. Newcastle disease virus primers NDVP1, NDVP2, product length 221 bp. The primers were synthesized by Nanjing Kingsray Biotechnology Co., Ltd. and stored at -20 °C.

3.3 Viral RNA Extraction

50 mg of the sample to be inspected was weighed and placed in a tissue grinder by a sterile operation, and the mixture was ground and ground, and after grinding for a while on ice, 1.5 mL of physiological saline was added, and the ground mixture was centrifuged. Take tissue microfilarum 300 μL in 1.5 mL EP tube, add 750 mL Trizol, mix, place at room temperature, and add 0.1 mL of chloroform after 5 min. Then, the liquid was mixed, placed at room temperature for 10 min, centrifuged at 12000 rpm for 15 min at 4 °C, and centrifuged, and about 2 mL of the supernatant was placed in a centrifuge tube. Add 500 μL of isopropanol, shake well and mix well. Leave at room temperature for 10 min, continue centrifugation for 10 min, remove the supernatant, wash the precipitate with ethanol solution and separate the precipitate. Thereafter, 10 μL of RNase-free trihydrogenated water treated with DEPC was used to dissolve the precipitate, and then 0.5 μL of HRP RNA inhibitory enzyme was added to the solution for RT-PCR detection[4].

3.4 Single RT-PCR Amplification

RT-PCR amplification of four primers for a single primer-RNA was extracted with four mixed antigens, and RT-PCR amplification was performed using primers FP1/FP2, P3/P4, P5/P6, and NDVP1/NDVP2, respectively.

3.5 Multiple RT-PCR Reactions

The multiple PCR reaction was carried out using the H5, H7, AND H9 subtype avian influenza viruses and New-

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castle disease virus as templates and after the reaction was completed, the amplified product was detected by agarose gel electrophoresis. The primer ratio, primer concentration, reaction temperature and time were repeatedly optimized to determine the reaction system of H5, H7, AND H9 subtype avian influenza viruses and Newcastle disease virus.

3.6 Specificity Test

The multiple PCR reaction of the RNA of H5, H7, and H9 subtype avian influenza viruses, Newcastle disease virus, Duck tembusu virus (DTMUV), avian infectious laryngotracheitis virus (ILTV), avian infectious bronchitis virus (IBV), and Egg drop syndrome virus (EDS) were carried out using the designed reaction system to verify the specificity of the constructed system.

3.7 Sensitivity Test

The multiple RT-PCR reaction was carried out by 10-fold dilution of H5, H7, AND H9 subtype avian influenza virus and Newcastle disease virus RNA, and the sensitivity of the one-step multiple RT-PCR to the minimum detection amount of RNA was determined.

4. Results

4.1 Single RT-PCR Results

RNA was extracted with four mixed antigens, and then amplified by RT-PCR with primers FP1/FP2, P3/P4, P5/P6 and NDVP1/NDVP2, respectively, and positive bands were amplified, as shown in Figure 1.

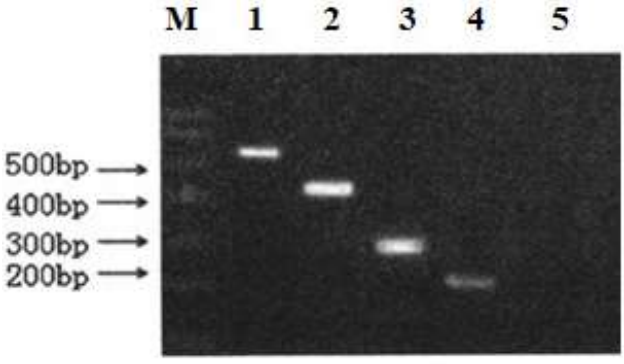

Figure 1. Single RT-PCR results

4.2 Establishment of Multiple RT-PCR Reaction System

The multiple RT-PCR reaction was carried out using H5, H7, AND H9 subtype avian influenza virus and Newcastle disease virus as templates, and after the reaction was completed, the amplified product was detected by agarose gel electrophoresis. After repeated optimization of primer ratio, primer concentration, reaction temperature and time, the reaction system of H5, H7, and H9 subtype avian influenza virus and Newcastle disease virus can be determined: The total reaction system is 25 μL. The system contains 15 μL of double distilled distilled water, 2 μL of magnesium chloride solution, 2.5 μL of PCR Buffer, 2 μL of template cDNA and 0.25 μL of EX TAQ, P1 (or P2, P3, P4, P5, P6, NDVP1, NDVP2) 4 μL, dNTP. The reaction system was placed in a 90 °C environment for 5 min, and then denatured at 94 °C for 1 min, annealed at 55 °C for 50 s, extended at 72°C for 1 min, 30 cycles, and extended at 72 °C for 10 min.

4.3 Specificity Test

The multiple PCR reaction was carried out with the designed reaction system using H5, H7, H9 subtype avian influenza virus, Newcastle disease virus, Duck tembusu virus (DTMUV), avian infectious laryngotracheitis virus (ILTV), avian infectious bronchitis virus (IBV), and Egg drop syndrome virus (EDS) RNA to verify the specificity of the constructed system. The results showed that only the H5, H7, H9 subtype avian influenza virus, Newcastle disease virus template as a template can amplify the target band and the other cannot amplify the corresponding band, as shown in Figure 2.

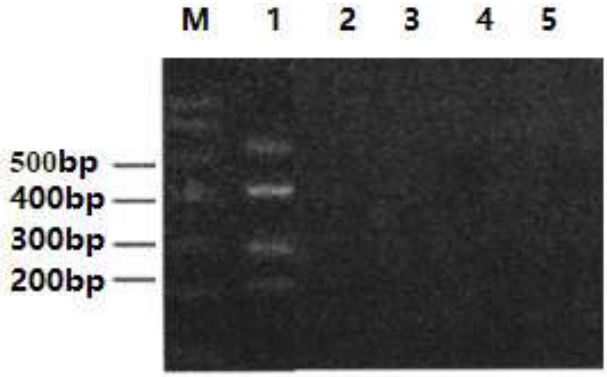

Figure 2. Specificity test results

Notes: M: Marker, 1: H5, H7, and H9 subtype avian influenza virus, Newcastle disease virus, 2: DTMUV, 3: ILTV, 4: IBV, 5: EDS

4.4 Sensitivity Test

The multiple RT-PCR reaction was carried out by 10-fold dilution of H5, H7, and H9 subtype avian influenza virus and Newcastle disease virus RNA, and the sensitivity of the one-step multiple RT-PCR to the minimum detection amount of RNA was determined. The test results show that H5, H7, H9 subtype avian influenza virus and Newcastle disease virus can reach 10^{-4}, and the sensitivity of

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H9 subtype avian influenza virus can reach 10^{-6}.

5. Discussion

5.1 Necessity of Multiple RT-PCR Detection of Avian Influenza and Newcastle Disease Virus

Both avian flu and Newcastle disease are avian infectious diseases caused by viruses, which pose a great threat to aquaculture and human health and have been designated as Class A infectious diseases by the International Office of Epizootics, and included in the list of animal infectious diseases of the International Biological Weapons Convention. The incident of bird flu infection in Hong Kong in the 1990s caused panic and highlighted the need for avian flu prevention.

Although there have been many reports on multiple RT-PCR detection methods for H5, H7 and H9 subtype avian influenza, however, since H3 subtype AIVs derived from ducks are prevalent in chickens in China and have been isolated from chickens in China and other countries, the detection data and methods related to other countries are highly biased. The mature method is not fully applicable to China's national conditions, and there are also fewer methods for distinguishing H5, H7, H9 subtype AIVs and NDVs, therefore, it is necessary to establish a scientific, flexible, rapid, convenient and accurate detection method to detect and distinguish these diseases[5].

5.2 Inadequacies and Disadvantages of Current Detection Methods

HA and HI tests, virus isolation and identification, virus neutralization test (VNT), enzyme-linked immunosorbent assay (ELISA) avian influenza, NIT test, immunofluorescence technique (IFT), agar gel diffusion test (AGP) are Newcastle disease Commonly used differential diagnostic techniques. The application of these techniques often requires a long time to obtain accurate test results, making it difficult to perform rapid differential diagnosis. Moreover, the virus content in animal products is low, and it is difficult to detect whether or not a virus is present by the above technique. If a simple PCR reaction is used, multiple PCR reactions are required to detect multiple viruses, which is laborious and costly[6].

5.3 Advantages of This Method

The multiple RT-PCR rapid detection method takes only a few hours, but the conventional detection method often takes several days, and the multiple RT-PCR detection method costs less, the operation is simpler, and the equipment requirements are not high, therefore, multiple RT-PCR technology as a rapid detection technology has a wide range of applications in clinical detection[7]. This experiment combined with relevant literature and case to screen four pairs of specific primers, which can be quickly distinguished in the same reaction system and can accurately diagnose suspected infection cases. The RNA mixed with the virus antigen was extracted, and four pairs of primers were used for multiple RT-PCR, and the results showed three clear target bands of different sizes. It is indicated that simultaneous detection of four viral antigens in the same system is feasible. Consistent with the virus isolation and identification results, the time is only 4 hours. This multiple RT-PCR method has shown great advantages in the differential diagnosis of suspected cases. It is of great practical significance to rapidly differentiate and diagnose H5, H9 subtype AIV and NDV infections in clinical diagnosis[8].

6. Conclusion

The avian influenza virus has the characteristics of high mortality, strong contagiousness and serious harm, which will bring a very heavy blow to the aquaculture industry, therefore, it is very necessary to strengthen the research and prevention of avian influenza virus. Because the avian influenza virus has many subtypes and is easy to mutate, it brings great difficulty to the identification and identification of viruses. The traditional detection method has a long duration, high cost, insufficient detection precision, and it is difficult to meet the needs of current disease prevention and control. Therefore, this study is mainly aimed at the identification and identification methods of subtype avian influenza virus and Newcastle disease virus. By using a variety of RT-PCR detection methods, it can quickly and accurately identify the virus type and create a good environment for avian influenza prevention and treatment.

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