Preparation and evaluation of combined vaccine against necrotic enteritis and colibacillosis in chickens and detection of maternal immunity in their progeny

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ABSTRACT

Necrotic enteritis (NE) caused by Clostridium perfringens type A and colibacillosis caused by Avian pathogenic E. coli (APEC), are two pathogenic diseases that threaten the poultry industry worldwide. A combined inactivated vaccine from Clostridium perfringens type A toxoid and serotypes O1 and O78 of E-coli adjuvanted with montanide gel was prepared and evaluated in two weeks old SPF white Lohman layer chickens and its progeny. The prepared vaccine was found safe and produced antitoxic titre against NE of 10 IU after 22 week of vaccination as measured by serum neutralization test and 2692 ELISA titre. Also it produced a humeral antibody titre against E.coli serotypes used of 80 at the 22th week post vaccination by microagglutination test (MAT) and an 80% protection in challenge against virulent E.coli serotypes used. Conclusion: vaccination of chicken with two doses, 3 weeks apart, of combined vaccine of Clostridium perfringens type A toxoid and serotypes O1 and O78 of E-coli adjuvanted with montanide gel, could protect against necrotic enteritis and colibacillosis.

Keywords: Necrotic enteritis- Colibacillosis - Clostridium perfringens-vaccine-chicken

1. INTRODUCTION

Necrotic enteritis (NE) is a disease of broilers and hens either breeders or layers at different ages starting from two weeks until twelve weeks old. Clostridium perfringens type A is the most common cause of poultry NE because it produces alpha toxin which has long been considered as a major factor in pathogenesis of NE (Cooper et al., 2010).

Avian pathogenic E.coli (APEC) is one of the most important opportunist pathogens in industrialized poultry production. APEC is associated with a variety of extra intestinal disease syndromes. In young chicks, APEC-induced omphalitis (yolk sac infection) which may result in generalized septicemia with perihepatitis, whereas respiratory infection may occur after aerogenic transmission (Pires dos Santos et al., 2013).

Colisepticemia usually occurs among birds with 2 to 12 weeks of age, with the majority of the cases occurring among birds
with 4 to 9 weeks of age with mortality reaching rates as high as 20% (Dho-Moulin and Fairbrother, 1999).

A vaccine for NE of chicken would reduce the need to prevent or treat the disease in broiler chickens with antibacterial drugs (Thompson et al., 2006).

With increased regulation on use of antibiotics to prevent infections and antibiotic resistance in bacteria, especially E.coli, alternatives are needed to improve animal health and welfare, such as new vaccines (Mellata, 2013).

One day old chicks has ill developed immune system depending mainly on maternal derived immunity and become sensitive to different pathogens, moreover this ill developed immune system resulting in weak antibody response to vaccinal program, this problem affects mass parenteral vaccination at day one which need enhancing immune response as it is not applicable (Mot et al., 2014).

Controlling of these diseases by active immunization is of considerable importance for better control over the disease spread and ultimately eradication of NE & Colibacillosis diseases in chickens with minimum cost as combined vaccines have the advantage of protecting against more than one disease at the same time, beside, reducing vaccination expenses, number of vaccination performed and saving time.

So the aim of the present work was to prepare a combined inactivated vaccine from Clostridium perfringens type A toxoid and serotypes O1 and O78 of E.coli adjuvanted with montanide gel for improving its immunogenicity. Also, evaluating the protective efficacy of the prepared vaccines by SNT and ELISA for Clostridium perfringens type A and E.coli serotypes O1 and O78(organism and antibodies). They were fed on free balanced ration, and used for evaluation of prepared vaccines.

2.2.3. Mice:

A total number of 100 swiss mice, were used for determination of safety tests for the prepared vaccines and serum neutralization test (SNT).

2.3. Vaccine preparation:

2.3.1. Necrotic enteritis vaccine

Clostridium perfringens type A toxoid was prepared according to Ahmed (1975). 0.5 ml of vaccine (equal to 1 vaccinal dose) was adjusted to contain 100 MLD of C.
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perfringens alpha toxoid. Then montanide gel was added (SEPPIC®, France) in a ratio of 20 adjuvant: 80 antigen.

2.3.2. Colibacillosis vaccine: was prepared according to Chaffer et al., (1997)

E.coli strains serotypes O₁ and O₇₈ were grown separately onto brain heart agar in Roux bottles and incubated at 37°C for 24 hr. The colonies were collected using normal saline then mixed together and bacterial suspension was adjusted to be 1x10⁹ CFU/0.5 ml (vaccinal dose). The bacteria were then inactivated by adding 0.5% formalin with agitation then montanide gel (SEPPIC®, France) was mixed with bacterial suspension in a ratio of 20 adjuvant: 80 antigen.

2.3.3. Combined vaccine against necrotic enteritis and Colibacillosis:

A combined vaccine from Clostridium perfringens type A toxoid (100 MLD of C. perfringens alpha toxoid/dose) and inactivated E.coli strains serotypes O₁ and O₇₈ (1x10⁹ CFU/dose) adjuvanted with montanide gel (SEPPIC®, France) was prepared; the vaccinal dose was adjusted to be 0.5 ml. All vaccines were subjected to safety and sterility test before immunization according to British Pharmacopeia (2010) and OIE (2017).

2.4. Experimental design:

Chickens were divided into 4 groups, 40 for each group as follow:

Group (1): was vaccinated with necrotic enteritis vaccine.

Group (2): was vaccinated with Colibacillosis vaccine.

Group (3): was vaccinated with combined vaccine.

Group (4): was left as control (inoculated with 0.5 ml saline).

Each chicken in first, second and third group was inoculated with 0.5 ml of each vaccine S/C in the middle dorsal back of neck two doses with 3 weeks interval for each vaccine. Serum samples were collected regularly before immunization, 2, 3, 6, 10, 14, 18, 22 weeks after 1st vaccination. Sera of each group were pooled and kept at -20°C until used for evaluating the developed humoral immune response.

-Hatched chicks were collected from eggs of vaccinated hens at 22 weeks of age. Then they were bled for serum collection at day one and day three.

2.5. Quality control testing of the prepared experimental vaccines:

2.5.1. Sterility test:

The prepared vaccines were tested to be free from any contaminant (aerobic and anaerobic bacteria, fungus and mycoplasma according to OIE (2017).

2.5.2. Safety test:

Safety of the prepared vaccines was tested according to OIE (2017); 10 chicks one day old were injected subcutaneously with double field dose of the prepared vaccines. The inoculated chicks were observed for 14 successive days to detect any signs of local or systemic reaction.

2.5.3. Determination of immune response against the prepared vaccines:

2.5.3.1. Serological evaluation of humeral immune response of the vaccinated chickens against Clostridium perfringens type A:

2.5.3.1.1. Antitoxin assay by SNT:

Pooled sera of each group were tested for determination of the alpha antitoxin titer of Clostridium Perfringens type A using SNT in mice according to Gadalla et al. (1971)

2.5.3.1.2. Antitoxin assay by ELISA:

ELISA was performed on the serum sample, according to the method described by Voller et al. (1976) and Briggs and Skeels (1984). The results were calculated according to the following formula:
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S/p (sample/positive)  
\[ \frac{\text{sample mean-negative control}}{\text{positive control-negative control}} \]  
\[ \log_{10} \text{titre} = 1.08(\log_{10} \text{S/P}) + 3.82 \]  
Titre = Anti-\log_{10}

2.5.3.2. Serological evaluation of humeral immune response of the vaccinated chickens against E coli serotypes O\textsubscript{1} and O\textsubscript{78}:

2.5.3.2.1. Micro-agglutination test (MAT):

Antibody response in vaccinated and unvaccinated chickens was followed up on regular intervals post vaccination determined by Micro-agglutination test (MAT) using sonicated antigen, according to the method described by Thaxton \textit{et al.} (1970) and Brown \textit{et al.} (1981)

2.5.3.2.2. ELISA:

ELISA was performed on the same serum sample, according to the method described by Voller \textit{et al.} (1976) and Briggs and Skeels (1984). The results were calculated according to the following formula:

\[ \frac{\text{sample mean-negative control}}{\text{positive control-negative control}} \]  
\[ \log_{10} \text{titre} = 1.09(\log_{10} \text{S/P}) + 3.63 \]  
Titre = Anti-\log_{10}

2.5.4. Challenge test:

Three weeks post-boostering, group 2 and 3 were subdivided into two subgroups; First subgroup were injected into the thigh region with 0.2 ml containing \( 10^7 \) CFU of E.coli serotype O\textsubscript{1} and the second subgroup were injected with E.coli serotype O\textsubscript{78} and monitored for clinical signs. Mortality was recorded for 7 days after challenge according to the method described by Chaffer \textit{et al.} (1997).

3. RESULTS

The prepared vaccines were found to be safe and sterile during the period of observation.

The results of SNT table (1) showed that the antitoxin titer of chickens vaccinated with \textit{Clostridium perfringens} type A only showed increasing antibody titer begins with 15 IU 2 weeks after vaccination, reached peak (25 IU) at the 10\textsuperscript{th} week post vaccination, then decreased and reached 10 IU after 22 weeks. While the antitoxin titer of chickens vaccinated with combined vaccine begins with 10 IU 2 weeks post vaccination, then reached the peak (20 IU) 10 weeks post vaccination and declined till reaching 10 IU at the 22\textsuperscript{th} week post vaccination.

ELISA titers in table (2) against \textit{Clostridium perfringens} type A alpha toxin in serum of vaccinated chickens came in parallel with the SNT results.

The humeral immune response against E.coli serotypes O\textsubscript{1} and O\textsubscript{78} was measured by ELISA as shown in tables (5, 6). For O\textsubscript{1} antigen the titer start with 850 and 870 at 2\textsuperscript{nd} week post first vaccination then increased and reached peak at 6\textsuperscript{th} week to 320 then start decreasing at 18\textsuperscript{th} week and reached 80 at 22\textsuperscript{th} week for both vaccines.

Also the humeral immune response against E. coli serotypes O\textsubscript{1} and O\textsubscript{78} was measured by ELISA as shown in tables (5, 6). For O\textsubscript{1} antigen the titer start with 920 and 942 at 2 week post to the method described by Chaffer \textit{et al.} (1997).

The results of SNT table (1) showed that the antitoxin titer of chickens vaccinated with \textit{Clostridium perfringens} type A only showed increasing antibody titer begins with 15 IU 2 weeks after vaccination, reached peak (25 IU) at the 10\textsuperscript{th} week post vaccination, then decreased and reached 10 IU after 22 weeks. While the antitoxin titer of chickens vaccinated with combined vaccine begins with 10 IU 2 weeks post vaccination, then reached the peak (20 IU) 10 weeks post vaccination and declined till reaching 10 IU at the 22\textsuperscript{th} week post vaccination.

ELISA titers in table (2) against \textit{Clostridium perfringens} type A alpha toxin in serum of vaccinated chickens came in parallel with the SNT results.

The humeral immune response against E. coli serotypes O\textsubscript{1} and O\textsubscript{78} was measured by ELISA as shown in tables (3, 4). It was clear that for both antigens the titer start with 40 at 2\textsuperscript{nd} week post first vaccination then increased and reached peak at 6\textsuperscript{th} week to 320 then start decreasing at 18\textsuperscript{th} week and reached 80 at 22\textsuperscript{th} week for both vaccines.

Also the humeral immune response against E. coli serotypes O\textsubscript{1} and O\textsubscript{78} was measured by ELISA as shown in tables (5, 6). For O\textsubscript{1} antigen the titer start with 920 and 942 at 2 week post to the method described by Chaffer \textit{et al.} (1997).

On the other hand, for O\textsubscript{78} antigen the titer starts with 920 and 942 at 2 week post first vaccination then increased and reached peak at 10\textsuperscript{th} week to 4345 and 4593 then start decreasing at 18\textsuperscript{th} week and reached 3479 and 3550 at 22 week for E. coli vaccine and combined vaccine, respectively.

The protection rate in table (7) measured by challenge test was 80% for both vaccines (\textit{E. coli} vaccine & combined vaccine) in chickens.

Table (8) showed the SNT and ELISA titers of hatched chickens from vaccinated hen with combined vaccine. The SNT titer raised
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from 1U in day one to 1.5 U in day three, also ELISA titer increased from 1413 to 2455 in day three. Regarding antibody titers against E-Coli O1 and E-Coli O78, it was 40 U by MAT for both serotypes. While by ELISA there was a slight difference between the two serotypes.

Table (1): Mean antitoxin titer against Clostridium perfringens type A alpha toxin in serum of vaccinated chickens as measured by SNT

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean antitoxin Titre (expressed as IU)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevacc.</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Group (1)</td>
<td>0</td>
</tr>
<tr>
<td>Group (3)</td>
<td>0</td>
</tr>
<tr>
<td>Group (4)</td>
<td>0</td>
</tr>
</tbody>
</table>

Group (1): chickens vaccinated with necrotic enteritis vaccine adjuvanted with montanide gel.
Group (3): chickens vaccinated with inactivated combined vaccine adjuvanted with montanide gel.
Group (4): Control unvaccinated group

Table (2): ELISA titer against alpha toxin of C. perfringens in sera of vaccinated chickens

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean ELISA Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevacc.</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Group (1)</td>
<td>150</td>
</tr>
<tr>
<td>Group (3)</td>
<td>167</td>
</tr>
<tr>
<td>Group (4)</td>
<td>100</td>
</tr>
</tbody>
</table>

Table (3): Microagglutination titer in sera of vaccinated chickens against E. coli O1

<table>
<thead>
<tr>
<th>Groups</th>
<th>Microagglutination Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prevacc.</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Group (2)</td>
<td>0</td>
</tr>
<tr>
<td>Group (3)</td>
<td>0</td>
</tr>
<tr>
<td>Group (4)</td>
<td>0</td>
</tr>
</tbody>
</table>

Group (2): chickens vaccinated with inactivated E. coli vaccine adjuvant with montanide gel.
Group (3): chickens vaccinated with inactivated combined vaccine adjuvant with montanide gel.
Group (4): Control unvaccinated group
Khalaf et al. (2018) (BVMJ 35(2): IN PRESS)

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Table (4) Microagglutination titer in sera of vaccinated chickens against E-Coli O\textsubscript{78}

<table>
<thead>
<tr>
<th>Groups</th>
<th>Microagglutination Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weeks post vaccination</td>
</tr>
<tr>
<td></td>
<td>Prevacc. 2 3 6 10 14 18 22</td>
</tr>
<tr>
<td>Group (2)</td>
<td>0 40 80 320 640 320 320 80</td>
</tr>
<tr>
<td>Group (3)</td>
<td>0 40 80 320 320 320 160 80</td>
</tr>
<tr>
<td>Group (4)</td>
<td>0 0 0 0 0 0 0 0</td>
</tr>
</tbody>
</table>

Group (2): chickens vaccinated with inactivated \textit{E. coli} vaccine adjuvant with montanide gel.
Group (3): chickens vaccinated with inactivated combined vaccine adjuvant with montanide gel.
Group (4): Control unvaccinated group

Table (5) ELISA titer in sera of vaccinated chickens against E.coli serotype O\textsubscript{1}

<table>
<thead>
<tr>
<th>Groups</th>
<th>ELISA Antibody Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weeks post vaccination</td>
</tr>
<tr>
<td></td>
<td>Prevacc. 2 3 6 10 14 18 22</td>
</tr>
<tr>
<td>Group (2)</td>
<td>96 850 2412 4150 4345 4150 3818 3479</td>
</tr>
<tr>
<td>Group (3)</td>
<td>117 870 2755 4217 4593 4217 3890 3550</td>
</tr>
<tr>
<td>Group (4)</td>
<td>100 120 240 166 188 166 157 100</td>
</tr>
</tbody>
</table>

Group (2): chickens vaccinated with inactivated \textit{E. coli} vaccine adjuvant with montanide gel.
Group (3): chickens vaccinated with inactivated combined vaccine adjuvant with montanide gel.
Group (4): Control unvaccinated group

Table (6) ELISA titer in sera of vaccinated chickens using O\textsubscript{78} antigen

<table>
<thead>
<tr>
<th>Groups</th>
<th>ELISA Antibody Titre</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weeks post vaccination</td>
</tr>
<tr>
<td></td>
<td>Prevacc. 2 3 6 10 14 18 22</td>
</tr>
<tr>
<td>Group (2)</td>
<td>111 920 2532 3960 4371 4205 3731 3264</td>
</tr>
<tr>
<td>Group (3)</td>
<td>123 942 2675 4078 4403 4327 3780 3378</td>
</tr>
<tr>
<td>Group (4)</td>
<td>114 125 140 166 173 182 167 130</td>
</tr>
</tbody>
</table>

Group (2): chickens vaccinated with inactivated \textit{E. coli} vaccine adjuvant with montanide gel.
Group (3): chickens vaccinated with inactivated combined vaccine adjuvant with montanide gel.
Group (4): Control unvaccinated group

Table (7): Results of challenge test with E-coli serotypes O\textsubscript{1} and O\textsubscript{78} among chickens vaccinated with the prepared vaccines

<table>
<thead>
<tr>
<th>Groups</th>
<th>Total No. of challenged birds</th>
<th>No. of dead birds / Total birds</th>
<th>Protection rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group (2)</td>
<td>20</td>
<td>2/10 2/10</td>
<td>80%</td>
</tr>
<tr>
<td>Group (3)</td>
<td>20</td>
<td>2/10 2/10</td>
<td>80%</td>
</tr>
<tr>
<td>Group (4)</td>
<td>20</td>
<td>8/10 2/10</td>
<td>20%</td>
</tr>
</tbody>
</table>

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Table (8): specific IgG titers in the sera of hatched chicks by SNT and ELISA

<table>
<thead>
<tr>
<th>Test</th>
<th>Day one old</th>
<th>Day three old</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNT</td>
<td>1 U</td>
<td>1.5 U</td>
<td>0 U</td>
</tr>
<tr>
<td>ELISA</td>
<td>1413</td>
<td>2455</td>
<td>631</td>
</tr>
</tbody>
</table>

Table (9): Antibody titers in the sera of hatched chicks by microagglutination test (MAT) and ELISA

<table>
<thead>
<tr>
<th>Test</th>
<th>Serotypes</th>
<th>Day one old</th>
<th>Day three old</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT</td>
<td>O1</td>
<td>40</td>
<td>40</td>
<td>0 U</td>
</tr>
<tr>
<td></td>
<td>O78</td>
<td>40</td>
<td>40</td>
<td>0 U</td>
</tr>
<tr>
<td>ELISA</td>
<td>O1</td>
<td>1775</td>
<td>1805</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>O78</td>
<td>1690</td>
<td>1710</td>
<td>115</td>
</tr>
</tbody>
</table>
4. DISCUSSION

Necrotic enteritis represents one of the most important diseases that threaten poultry population (Tripathy and Reed, 2008), it causes severe economic losses by increasing mortality rates.

It was well known that vaccination is the cornerstone in controlling infectious diseases and it is known that flocks with high titers of maternal antibodies against alpha toxin had lower mortality during the production period than flocks with low titers (Heier et al., 2001).

Inactivated vaccines based on formalin or heat inactivated E. coli are generally believed to confer protection against avian Colibacillosis in an antibody-dependent manner (Arp, 1980 and Leitner et al., 1990). Because cross-protection is usually not observed with E. coli serotypes from poultry (Arp, 1980), a suitable vaccine would have to contain the most common serotypes.

So, the present work was to prepare a combined inactivated vaccine from *Clostridium perfringens* type A toxoid and serotypes O_1 and O_78 of E-coli adjuvanted with montanide gel for improving its immunogenicity. The study also evaluated the protective efficacy of the prepared vaccines by challenge test and evaluation of the immunizing and protective values of the prepared vaccines by SNT and ELISA For *Clostridium perfringens* type A and microagglutination test and ELISA for E.coli.

The results of SNT showed that the antitoxin titer of chickens vaccinated with two doses of *Clostridium perfringens* type A toxoid only showed increasing antibody titer begins with 15 IU and reached 10 IU after 22 weeks. While the antitoxin titer of chickens vaccinated with combined vaccine begins with 10 IU and reached the peak (20 IU) then declined to 10IU at the 22\textsuperscript{th} week post vaccination. The protective antibody titer against the alpha toxin of *Clostridium perfringens* is reported to be 0.5 IU/ml for alpha toxin (British Pharmacopeia, 2010). As egg production starts approximately at the 16-18 week of age in chicken, the antibody titer present is sufficient to confer immunity.

ELISA procedure is a rapid, specific and sensitive serological assay which has been used for the detection of several bacterial toxins.ELISA results come in parallel with the results of SNT. These results agreed with Heier et al (2001) and Lovland et al (2004), who showed that the vaccination of chickens with NE vaccine resulted in strong and specific antibody against alpha toxin of *Clostridium perfringens* type A.

Several authors have reported the isolation of E.coli strains O_1 and O_78 as one of the prevalent serotypes in Egypt as Abd El Tawab et al. (2016), Younis et al. (2017) and Moawad et al. (2018). so, these two strains were selected. The humeral immune response against E.coli serotypes O_1 and O_78 was measured by MAT and ELISA as illustrated in tables 3,4. it was clear that for both antigens the titer start rising at 2\textsuperscript{nd} week post first vaccination then increased and reached peak at 6\textsuperscript{th} week to 320 then start decreasing at 18\textsuperscript{th} week to reach 80 by the 22\textsuperscript{th} week for both vaccines.

Also the humeral immune response against E.coli serotypes O_1 and O_78 was measured by ELISA as shown in tables 5 and 6.
For O₁ antigen the titer start with 850 and 870 at 2ⁿᵈ week post first vaccination then increased and reached peak at 10ᵗʰ week to 4345 and 4593 then start decreasing at 18ᵗʰ week and reached 3479 and 3550 at 22ᵗʰ week for E-coli vaccine and combined vaccine, respectively. On the other hand, for O₇₈ antigen the titer starts with 920 and 942 at 2ⁿᵈ week post first vaccination then increased and reached peak at 10ᵗʰ week to 4371 and 4403 then start decreasing at 18ᵗʰ week and reached 3264 and 3378 at 22ᵗʰ week for E-coli vaccine and combined vaccine, respectively. These results agree with that obtained by Sadeyen et al. (2015) and El Jakee et al. (2016) who found that inactivated APEC vaccines are protective against experimental intra-airsac challenge in a turkey model of acute colibacillosis and that they both induce predominantly a Th2 response in the spleen that correlates with elevated APEC-specific antibody levels.

The protection rate measured by challenge test was 80% for both vaccines (E. coli vaccine & combined vaccine) in chickens and these results agreed with El Jakee et al. (2016).

Maternal antibody were detected in hatched chicks, as shown in table (8 and 9), the chicks have protective levels of both antigens in day one and day three. These results come as Lovland et al. (2004) who reported a strong serum immunoglobulin G response to C. perfringens alpha-toxin in parent hens, and specific antibodies were transferred to their progeny. Also, these results are in agreement with Heller et al. (1990) who stated that at hatching, the level of maternal antibody to E. coli in chicks, measured by ELISA was found to be 55 to 62% of that of the hen. It declined to an undetected level at 21 days of age.

From results obtained in this study it could be concluded that vaccination of chicken with combined vaccine of E coli and Clostridium perfringens type A vaccine is recommendable to be used for better controlling and eradication of necrotic enteritis and colibacillosis in chickens.


OIE Terrestrial Manual (2017): Chapter 1.1.9 Tests for sterility and freedom from contamination of biological materials.


