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Correlation between viable count and protection rates provided by live bacterial vaccines in chickens

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Abstract
Background: Colibacillosis is one of the most important diseases of the poultry industry around the world. It causes considerable economic damage every year. *Salmonella* Enteritidis and *Salmonella* Typhimurium are important cause of food-borne illness. Vaccination plays an important role in the overall bio-security system. This study aimed to the use of enumeration of viable bacterial count as an alternative method to challenge test to quantify the colony-forming units (CFU) of specified live bacterial poultry vaccines.
Material and methods: Two different live vaccinal mutant (*aroA* gene deleted *E. coli* O78 and *aroA* gene deleted *S.* Typhimurium STM-1 vaccines) were used in this study. The bacterial count was applied in each vaccine and different doses for each vaccine above and below the vaccinal dose were calculated and adjusted then inoculated in different groups of one day old SPF chicks and observed for 3 weeks for safety and shedding of the vaccinal organisms then challenged with the virulent strains of *E. coli* O78 and *S.* Typhimurium at 4th week post vaccination.
Result: Protection percent of *E. coli* vaccine ranged from 62% to 88% in groups inoculated with different doses while it was 16% in the unvaccinated chicken control group. While it was 66% to 84% in *S.* Typhimurium vaccine in different vaccinated groups while it was 12% in the unvaccinated chicken control group.
Conclusion: Results of this study stated that the maximum release titer for *aro*A gene deleted *E. coli* vaccine was 5x10^{8} CFU/dose while it was 1x10^{9} for *aro*A gene deleted *S.* Typhimurium vaccine. On the other hand, the minimum release titer was 5x10^{6} for *aro*A gene deleted *E. coli* vaccine while it was 1x10^{6} for *aro*A gene deleted *S.* Typhimurium vaccine.

Keywords: *E. coli*, S. typhimurium, live vaccine, PCR and chickens

Introduction
Enterobacteriaceae are a large family of gram negative bacteria that includes many of the more familiar pathogens that colonize the small and large intestine such as *E. coli* and *Salmonella* (Mellen *et al*., 2014) [1]. Colibacillosis is one of the most important diseases of the poultry industry around the world. It causes considerable economic damage every year, due to directly increased mortalities and indirectly because of reduced weight gain, increased feed conversion rate and carcass condemnation (Saif, 2008) [2]. *S.* Enteritidis and *S.* Typhimurium are important causes of food-borne illness. Based on surveillance studies, the main vehicles of *S.* Enteritidis and *S.* Typhimurium infection include raw meat, eggs and poultry products (Thung *et al*., 2016) [3].
Due to the frequency of antimicrobial resistance and the number of resistance determinants in *Salmonella* and *E. coli* have raised markedly, vaccination plays an important role in the overall bio-security system on animal farms, typically chicken farms to prevent *E. coli* and *Salmonella* infections (Jawale *et al*., 2012) [4]. Veterinary vaccines must be safe, pure, potent, and effective. Potency for most animal health vaccines was demonstrated by means of vaccination in specific host and subsequent challenge of the vaccinates with the live pathogenic agent but animal welfare considerations, cost, assay duration, and assay variability have all been drivers for the animal health industry to find alternative assays. For many vaccines containing live antigens, these tests have been largely replaced by *in vitro* assays that titrates the live antigen content in the vaccines (CFR, 2016) [5].

The main target of this study was the use of enumeration of viable bacterial count as an alternative method to challenge test to quantify the colony-forming units (CFU) of specified live bacterial poultry vaccines. With additional considerations in developing appropriate *in vitro* assays include the correlation of the assay to host animal efficacy.

Material and Methods

Vaccines

1. 1Live *E. coli* vaccine. (*Aro* A gene deleted E. coli O78 live vaccine)
2. Live *Salmonella* Typhimuium vaccine (*Aro* A gene deleted ST, STM-1 strain).

Identity of appropriate target antigens

1. **DNA Extraction:** DNA templates of both vaccinal strains were extracted by using Isolate Genomic DNA Mini Kit. (Bioline, Cat. No. Bio-52032). The extracted genomic DNAs of both vaccines were fractionated on 0.7% agarose gel.
2. **Primers used:** Specific primers of *E. coli* and *S.* Typhimurium were used and illustrated in Table (1).

Table 1: Primers used for identification *of E. coli* and *Salmonella*.

Primer	Primer Sequence	Product	Reference
16s rRNA E. coli	F5-GACCTCGGTTTAGTTCACAGA-3	585 bp	Tonu *et al*., 2011 [6]
	R5-CACACGCTGACGCTGACCA-3		
S. Typhimurium	F5-TTGTTCACTTTTTACCCCTGA-3	401 bp	Alvarez *et al*., 2004 [7]
	R5-CCCTGACAGCCGTTAGATATT-3		

3. **Polymerase chain reaction:** 5 µl of genomic DNA, 12.5 µl of dream taq green master mix (Thermo scientific #K1081), 1 µl of each primer (10 pmole) and 5.5 µl of deionized water were added to 0.5ml microcentrifuge tubes. The amplification reactions were performed under following conditions: 94 °C for 4 min, then 29 cycles each at 94 °C for 1.5 min, 62 °C for 1.5 min and 72 °C for 2 min.; lastly 72 °C for 10 min in case of *E. coli* (Tonu *et al*., 2011) [6], while it was 95 °C for 2 min, then 30 cycles each at 95 °C for 1 min, 57 °C for 1 min and 72°C for 2min.; lastly 72 °C for 5 min in case of S. Typhimurium (Alvarez *et al* 2004) [7]. The PCR products were analyzed on 1% agarose gel.

Enumeration of viable bacterial count using spread surface plate method (Sadeyen *et al*., 2014) [8]

The vial of vaccine was rehydrated in the required amount of MRD (maximum recovery diluent) in a way that the test portion represented one dose of vaccine then thoroughly mixed. A range of ten-fold serial dilutions was used then two inoculums of 0.1ml/each were pipetted into the surface of separate TSA agar plates per each dilution. The inoculum was spread rapidly over the entire agar surface using a thin bent glass rod. The plates are incubated for 24-48 hours at 37 °C. Colonies were counted on each of two plates using bacterial colony counter.

The colonies were counted as an average of two plates inoculated with the selected dilution.

Number of colony forming unit (CFU)/ml = N x 10^n x 10 where N = no. of colonies on the plate at the selected dilution n.

The viable colony count for *E. coli* vaccine was $5x10^7$ CFU/dose while it was $1x10^8$ CFU/ dose for *S.* Typhimurium vaccine**.** Different doses for each vaccine above and below the vaccinal dose were calculated and adjusted

Experimental design

A total of 650 one day old SPF chicks were used. Half of these chicks were used to study the live *E. coli* vaccine as they were subdivided into equal 6 groups (50 each) named from E1 to E6. These chicks received the live *E. coli* vaccine orally in a concentration of $5x10^{10}$, $5x10^9$, $5x10^8$, $5x10^{7,}$ $5x10^6$ and $5x10^5$ CFU/dose while the rest 7^{th} group E7 (25 chicks) was kept as a control unvaccinated group. The same program was typically applied regarding the live *S.* Typhimurium vaccine but the vaccine concentrations used were $1x10^{10}$, $1x10^9$, $1x10^8$, $1x10^7$, $1x10^6$ and $1x10^5$ CFU/dose and the groups named from S1 to S6 respectively and the 7^{th} group was unvaccinated control group S7 (25 chicks). Shedding of the vaccinal organisms was followed for 3 weeks post vaccination. The fecal samples were processed and samples containing typical *E. coli* or *Salmonella* colonies were confirmed by PCR using and *S.* Typhimurium specific primers. Four weeks post vaccination, these vaccinated chicks were challenged with the virulent bacteria ($1x10^6$ CFU/dose *E. coli* O78 for *E. coli* vaccinated and control groups and $1x10^7$ CFU/dose *S.* Typhimurium for *Salmonella* vaccinated and control groups). Challenged birds were observed daily for 2 weeks and examined for abnormal clinical signs, mortalities and PM lesions with re-isolation of challenge strains from the affected birds. After the observation period, all rest birds were sacrificed and examined for postmortem lesions.

Results

Identity of vaccinal strains: In the present study, the identity of the tested live attenuated *E. coli* and *S.* Typhimurium vaccines was performed to identify the target antigens. Firstly, the genomic DNAs of both vaccines were extracted, fractionated on 0.7% agarose and the estimated size of the genomic DNA was more than 23kbp. The target antigen of *E. coli* vaccine was confirmed as *E. coli* by its specific primer giving rise to a PCR product of 585 bp as shown in Figure (1A). Also the antigen of *S.* Typhimurium vaccine was confirmed as *S.* Typhimurium by using its specific primer giving rise to a PCR product of 401 bp as shown in Figure (1B).

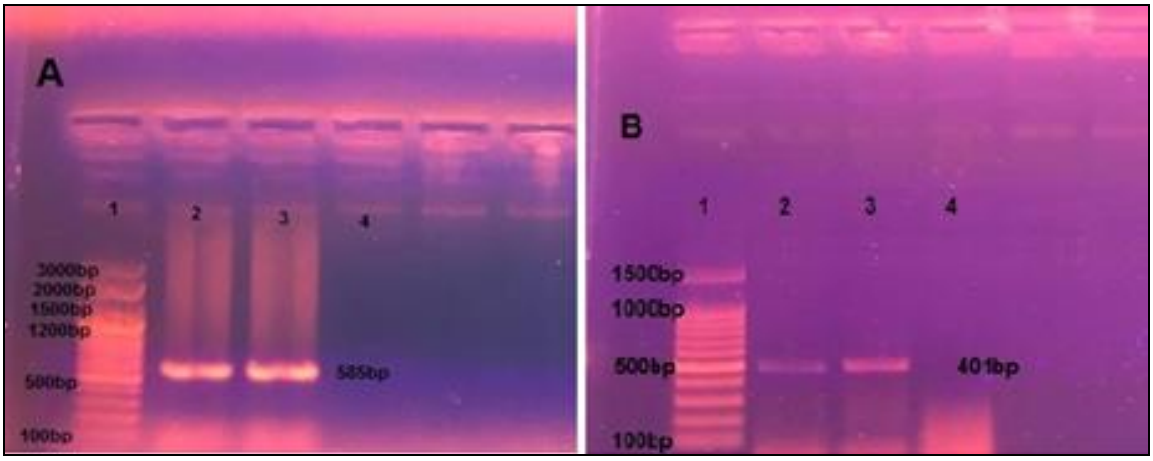

Fig 1: Result of identity of *E. coli* vaccine and *S. typhimurium* vaccine

Fig 1: (A) Result of identity of *E. coli* vaccine. Lane 1: marker, Lane 2: *E. coli* vaccine, lane 3: positive control and Lane 4: Negative control. (B) Result of identity of *S. typhimurium* vaccine. Lane 1: marker, Lane 2: *S.* Typhimurium vaccine, lane 3: positive control and Lane 4: Negative control.

Enumeration of viable bacterial count

The viable colony count for *E. coli* vaccine was $5x10^7$ CFU/dose and for *S.* typhimurium vaccine was $1x10^8$ CFU/dose**.**

Safety of different doses of used vaccines.

No mortalities or clinical signs attributable to the vaccine were observed in all groups during observation period except for subgroup E1 (received 10^{10}CFU/dose of the *E. coli* vaccine) where the mortality rate was 7 out of 50 birds and 11 birds showed depression, lower body weight and diarrhea as shown in Table (2).

Table 2: Safety of vaccination with different doses of both vaccines.

Type of vaccine	Subgroup	Dose (CFU/dose)	Mortality	Clinical signs	Safety
Aro A deleted *E. coli* vaccine	E1	$5x10^{10}$	7/50	11/50	Unsafe
	E2	$5x10^9$	0/50	0/50	Safe
	E3	$5x10^8$	0/50	0/50	
	E4	$5x10^7$	0/50	0/50	
	E5	$5x10^6$	0/50	0/50	
	E6	$5x10^5$	0/50	0/50	
Aro A deleted *S.* Typhimurium vaccine	S1	$1x10^{10}$	0/50	0/50	Safe
	S2	$1x10^9$	0/50	0/50	
	S3	$1x10^8$	0/50	0/50	
	S4	$1x10^7$	0/50	0/50	
	S5	$1x10^6$	0/50	0/50	
	S6	$1x10^5$	0/50	0/50	
Control	**E7 and S7**	**Non vaccinated**	-	-	-

Shedding of different doses of used vaccines

Vaccine strains were detected and identified by PCR in fecal swabs of vaccinated chickens up to 13 days post vaccination for all groups but by day 14 the organisms were eliminated as shown in Table (3). The fecal swabs were identified by PCR using *E. coli* and *S.* Typhimurium specific primers giving rise a PCR product of 585bp for *E. coli* vaccine as shown in Figure (2A) and 401 bp for *Salmonella* vaccine as shown in Figure (2B).

Table 3: Shedding of vaccinal strains

Type of vaccine	Subgroup	Dose (CFU/dose)	Shedding											
			1st	2nd	3rd	4th	5th	6th	7th	9th	11th	13th	14th	21th
Aro A deleted *E. coli* vaccine	E1	$5x10^{10}$	+	+	+	+	+	+	+	+	+	+	-	-
	E2	$5x10^9$	+	+	+	+	+	+	+	+	+	+	-	-
	E3	$5x10^8$	+	+	+	+	+	+	+	+	+	+	-	-
	E4	$5x10^7$	+	+	+	+	+	+	+	+	+	+	-	-
	E5	$5x10^6$	+	+	+	+	+	+	+	+	+	+	-	-
	E6	$5x10^5$	+	+	+	+	+	+	+	+	+	+	-	-
Aro A deleted S. Typhimurium vaccine	S1	$1x10^{10}$	+	+	+	+	+	+	+	+	+	+	-	-
	S2	$1x10^9$	+	+	+	+	+	+	+	+	+	+	-	-
	S3	$1x10^8$	+	+	+	+	+	+	+	+	+	+	-	-
	S4	$1x10^7$	+	+	+	+	+	+	+	+	+	+	-	-
	S5	$1x10^6$	+	+	+	+	+	+	+	+	+	+	-	-
	S6	$1x10^5$	+	+	+	+	+	+	+	+	+	+	-	-
Control	E7 and S7	Unvaccinated	-	-	-	-	-	-	-	-	-	-	-	-

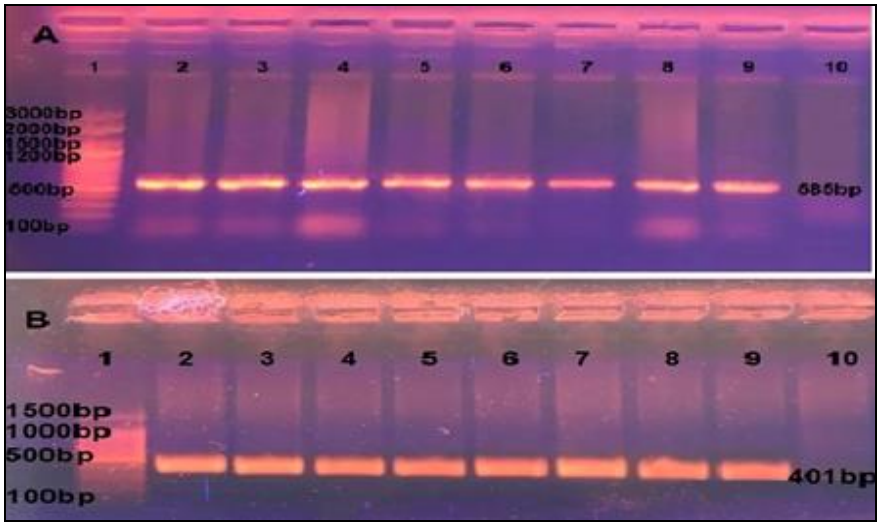

Fig 2: Results of shedding of *E. coli* vaccine and *S. Typhimurium* vaccine

Fig 2: (A) Results of shedding of *E. coli* vaccine. Lane 1: marker, Lane 2: positive control, lane 3 - 10: fecal swab at 1st 3rd, 5th, 7th, 9th, 11th, 13th, and 14th days post vaccination. (B) Results of shedding of *S.* Typhimurium vaccine. Lane 1: marker, Lane 2: positive control, lane 3 - 10: fecal swab at 1st 3rd, 5th, 7th, 9th, 11th, 13th, and 14th days post vaccination.

Post challenge immune status assessment

As regards to the protection percent obtained in chickens vaccinated with different doses of *E. coli* vaccine after challenge with *E. coli* O78 strain, the protection percent were 88, 84, 80, 74, 72 and 62% in subgroups E1, E2, E3, E4, E5 and E6 respectively while it was 16% in the unvaccinated chicken control group (E7) as shown in Table (4). On the other hand, the protection percent obtained in chickens vaccinated with different doses of *S.* Typhimurium vaccine after challenge with *S.* Typhimurium strain were 84, 80, 78, 74, 70 and 66% in subgroups S1, S2, S3, S4, S5 and S6 respectively while it was 12% in the unvaccinated chicken control group (S7) as shown in Table (5)

Table 4: Protective rates in chickens vaccinated with *E. coli* vaccine and challenged with *E. coli* O78 virulent strain.

***Aro* A gene deleted *E. coli* vaccine**							
Groups	**E1**	**E2**	**E3**	**E4**	**E5**	**E6**	**E7**
Vaccine doses	$5x10^{10}$	$5x10^{9}$	$5x10^{8}$	$5x10^{7}$	$5x10^{6}$	$5x10^{5}$	Control
Total No.	50	50	50	50	50	50	25
Mortality	1/50	1/50	3/50	5/50	6/50	9/50	12/25
Clinical Signs	2/50	3/50	3/50	4/50	4/50	4/50	6/25
PM lesions	3/50	4/50	4/50	4/50	4/50	6/50	3/25
Re-isolation	6/6	8/8	10/10	13/13	14/14	19/19	21/21
Totally affected	6/50	8/50	10/50	13/50	14/50	19/50	21/25
Protection %	88%	84%	80%	74%	72%	62%	16%

Table 5: Protective rates in chickens vaccinated with *S.* Typhimurium vaccine and challenged with *S.* Typhimurium virulent strain.

***Aro* A gene deleted *S.* Typhimurium vaccine**							
Groups	**S1**	**S2**	**S3**	**S4**	**S5**	**S6**	**S7**
Vaccine doses	$1x10^{10}$	$1x10^{9}$	$1x10^{8}$	$1x10^{7}$	$1x10^{6}$	$1x10^{5}$	Control
Total No.	50	50	50	50	50	50	25
Mortality	1/50	2/50	3/50	5/50	7/50	9/50	12/25
Clinical Signs	3/50	4/50	4/50	4/50	4/50	4/50	6/25
PM lesion	5/50	4/50	4/50	4/50	4/50	4/50	4/25
Re-isolation	8/8	10/10	11/11	13/13	15/15	17/17	22/22
Totally affected	8/50	10/50	11/50	13/50	15/50	17/50	22/25
Protection %	84%	80%	78%	74%	70%	66%	12%

Discussion

Colibacillosis is a serious disease of poultry caused by Avian Pathogenic *E. coli* (APEC) which is one of the most important causes of extra-intestinal diseases in the poultry industry including airsacculitis, pericarditis, perihepatitis and cellulitis. Colibacillosis results in significant economic losses to the poultry industry through mortality, morbidity, cost of treatment and condemnation at processing plant (Nagano *et al*., 2012) [9]. More than one hundred *E. coli* serotypes have been reported but the infection in chickens was mostly belonged to serogroups O_1, O_2 and O_{78} (Chart *et al*., 2000) [10].

Salmonellosis is an important disease of chicken all over the world. Avian host specific *salmonellae* include *Salmonella* Gallinarum (*S.* Gallinarum) and *Salmonella* Pullorum (*S.* Pullorum) which causes fowl typhoid and pullorum disease respectively (Rajagopal and Mini, 2013) [11]. By contrast, non-host-specific *Salmonella* like *Salmonella typhimurium or Salmonella enteritidis* are commensal in poultry and can persist in the gastrointestinal tract. They are mainly asymptomatic but are associated with widespread human illness. Food vehicles such as poultry meat, eggs, and poultry

by-products are among the most common sources of *Salmonella* infections to human (Dunkley *et al*., 2009) [12].

Live vaccines based on defined mutations that impair virulence and non-revering have been shown to reduce the virulence of *E. coli* and *Salmonella* strains. In the present study the identity of the tested live attenuated *E. coli* and *S.* Typhimurium vaccines were performed to identify the target antigens. Firstly, the genomic DNAs of both vaccines were extracted, fractionated on 0.7% agarose and the estimated size of the genomic DNA was more than 23 kbp. The target antigen of *E. coli* vaccine was confirmed as *E. coli* by using its specific primer giving rise to a PCR product of 585 bp. This result was confirmed by 585 bp region of *E. coli* chromosome which amplified from *E. coli* isolates identified by Khatun *et al*. (2015) [13] from naturally healthy broiler chickens in Bangladesh by PCR using the same primer and Tonu *et al*. (2011) [6] who used PCR to detect the pathogenic *E. coli* through pathological study of the colibacillosis affected birds.

Also the antigen of *S.* Typhimurium vaccine was confirmed as *S*. Typhimurium by using *S*. Typhimurium primer giving rise to a PCR product of 401 bp. The same PCR product obtained by Alvarez *et al*. (2004) [7] who developed a multiplex PCR technique for detection and epidemiological typing of *Salmonella* in human clinical samples.

The bacterial count was applied on each vaccine and the viable colony count for *E. coli* vaccine was $5x10^7$ CFU/dose and was $1x10^8$ CFU/ dose for *S.* Typhimurium vaccine. Different doses for each vaccine above and below the vaccinal dose were calculated and adjusted then inoculated in different groups of one day old SPF chicks and observed for 3 weeks for safety and shedding of the vaccinal organisms.

As regard to the safety in *E. coli* vaccinated birds, the vaccine administered at a dose of $5x10^9$, $5x10^8$, $5x10^{7,}$ $5x10^6$and $5x10^5$ was found to be safe and didn't cause any mortalities, clinical illness or affect growth rate while at a dose of $5x10^{10}$ the mortality was 7 out of 50 birds and 11 birds showed depression, lower body weight and diarrhea**.** Ahlam *et al*. (2017) [14] revealed that neither mortalities nor colibacillosis lesions were observed in 70 day old broiler chicks vaccinated with *aro* A mutant *E. coli* vaccine with a dose containing $5.2x10^6$- $9.1x10^8$ CFU/chick. Also Salehi *et al*. (2012) [15] confirmed the safety of *aro* A mutant *E. coli* vaccine administered at a dose of 10^8 CFU. These studies proved the safety of the maximum release titer of the vaccine ($5x10^8$) which means the expected highest number of viable *E. coli* allowed per dose and safety of its ten field dose ($5x10^9$).

On the other hand the administration of *S.* Typhimurium vaccine was found to be safe at all doses $1x10^{10}$, $1x10^9$, $1x10^8$, $1x10^7$, $1x10^6$ and $1x10^5$. Alderton *et al*., (1991) [16] concluded that the inoculation of aro A *S.* Typhimurium deletion mutant orally in day- old chicks at a doses of 10^9, $10^{9.7}$ and 10^{10} CFC/bird didn't adversely affect the weight gain of chickens, survived the experiment and showed no adverse reactions. Also Groves *et al*., (2016) [17] concluded that the safety of live attenuated *aro* A deletion mutant *S.* Typhimurium STM-1 strain vaccine at its recommended dose 10^8 CFU/dose.

From this study, the approved maximum release dose for aro A deleted mutant *S.* Typhimurium vaccine was 10^9 CFU/dose. Also inoculation of tenfold dose of MRD (maximum release dose) was found to be safe. The shedding of each vaccine organism was observed for 3 weeks post inoculation and organisms were found to be eliminated by day 14 post inoculation. So, the onset of immunity was demonstrated to be 14 days post vaccination and limited protection is expected prior to that point as stated also by EMA (2012) [18] for *E. coli* vaccine.

Alderton *et al*., (1991) [16] examined the fecal swabs 2, 5 and 14 days post inoculation of *S.* Typhimurium vaccine and concluded that all chickens were excreting the organism within 5 days but the organism was eliminated by day 14. On the contrast with Revolledo and Ferreira (2010) [19] who evaluated antibiotic- mutant strains of *S.* Typhimurium and stated that the fecal shedding persisted at 1st, 2nd and 3rd weeks. John (2015) [20] evaluated *S.* Typhimurium vaccine against *S.* Typhimurium, *S.* Enteritidis and *S.* Gallinarum infection and stated that the fecal shedding ended by day 10 post vaccinations.

Regarding the vaccination challenge assay for *E. coli* vaccine, the highest protection percent obtained with highest dose 10^{10} which reduced from 88% to be 62% with the dose of $5x10^5$. This finding conclude the efficacy of the vaccine in terms of its ability to reduce the colonization of virulent O78 strain compared by the control group that gave protection 16% only. Also the study demonstrated that the minimum vaccinal dose that gave the approved protection percent was $5x10^6$ CFU/dose. So, it could be concluded that the expected lowest number of viable organism required per dose in *E. coli* vaccine verified by efficacy (minimum release titer) was $5x10^6$CFU/dose. Ahlam *et al* (2017) [14] concluded that the cumulative collibacillosis rates one week after challenge were 28.6% in one day chicks vaccinated by aroA mutant vaccine with a dose containing $5.2x10^6$-9.1 $x10^8$ CFU/chick as compared by 93.3% in control group.

From the vaccination challenge assay for *S.* Typhimurium vaccine, the protection percent ranged from 84% to 66% with reduced doses. From this study, the minimum protective dose for Salmonella vaccine was 10^6CFU/dose. A preliminary study on the response to different dosages of STM-1 applied by Alderton *et al*. (1991) [16] showed that the higher vaccine doses (10^6 and 10^{10} CFU/bird) stimulated rapid and higher antibody response than a lower dose (10^4 CFU/bird). This may explain our results of higher protection with higher doses. Also Alderton *et al* (1991) [16] explained that oral vaccination at 10^8 caused a cessation in virulent STM excretion 35 days post challenge.

Finally, EMA 2012 [18] concluded that the approved titer range per dose is $5.2x10^6$ to $9.1x10^8$ CFU for aroA deleted *E. coli* vaccine and 10^6 to 10^9 CFU for *S.* Typhimurium vaccine. So, from the previous mentioned data, it could be concluded that the expected highest number allowed per dose (maximum release titer) for *aro* A gene deleted *E. coli* vaccine was $5x10^8$ CFU/dose while it was $1x10^9$ for *aro* A gene deleted *S.* Typhimurium vaccine. On the other hand, the minimum vaccine dose that gave the approved protection percent (minimum release titer) is $5x10^6$ for *aro* A gene deleted *E. coli* vaccine while it was $1x10^6$ for *aro* A gene deleted *S.* Typhimurium vaccine. Also the onset of immunity was demonstrated to be 14 days post vaccination and limited protection is expected prior to that point.

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