Short communication

Sodium dodecyl sulfate- cured nalidixic acid resistant 85-kb plasmid *Salmonella gallinarum* Immunoglobulin G response in brown layer hens

ABSTRACT

Aim: Fowl typhoid vaccination is a necessary complement to farm hygiene in reducing antimicrobial resistance caused by extensive prophylaxis of antibiotics in poultry. This study was undertaken to develop a vaccine candidate from a virulent strain by plasmid-curing.

Place and Duration of Study: The research was carried out in the Department of Pharmaceutical Microbiology and Biotechnology, University of Nigeria Nsukka, for six months

Methodology: Thirty day-old pullets were divided into three groups of ten birds each. This comprised a negative control group (unvaccinated) (NEG), a live SG9R vaccine positive group (SG9R), and a nalidixic acid resistant plasmid-cured **85-kb plasmid *Salmonella gallinarum (NAR).*** Plasmid curing of the virulent strain was done by incubating in sodium dodecyl sulfate (SDS) and loss of the 85-kb plasmid was identified and determined on agarose gel electrophoresis. Vaccination was done subcutaneously at 4 and 7 weeks of age, followed by challenge with the virulent *S. gallinarum.* IgG was measured using Enzyme-linked Immunosorbent Assay (ELISA). The scalability of the SDS-cured nalidixic acid resistant 85kb plasmid *Salmonella gallinarum* immunity was demonstrated by vaccinating layer birds and comparing the humoral immunity with that of a commercial fowl typhoid vaccine (SG9R).

Results: There were higher IgG levels in the NAR group than the SG9R group. Protection was above 70 % in the vaccinated groups.

Conclusion: The outcome of this present study shows that vaccination with viable cells of sodium dodecyl sulfate- cured nalidixic acid resistant 85-kb plasmid *Salmonella gallinarum* (NAR) provided layer birds with protective humoral immunity against subsequent challenge with the parent virulent strain containing the 85-kb plasmid.

Key words: Salmonella Gallinarum, plasmid, curing, immunoglobulin G, layer hens

1. INTRODUCTION

S. gallinarum produces acute septicemic fowl typhoid of poultry but rarely causes disease in man. It is host specific. Host specificity is expressed primarily at the level of the liver and spleen. It produces disease in both young and adult chickens [1]. Mortality can get as high as 100 % especially with newly hatched chickens [2]. The experimental *Salmonella enterica* serovar Gallinarum biovar Gallinarum was isolated from a case of fowl typhoid possessing an 85-kilobase plasmid [3,4] A work has shown that the very high virulence of *Salmonella gallinarum* is related to the possession of a high-molecular-weight (85-kilobase) plasmid [5,6,3]. From pathogenesis studies, the plasmid has been recognized to penetrate and spread *in vivo* and equipped the strain with adaptation mechanisms within the host environment. Several factors impact adaptability and these include the acquisition of genes through horizontal gene transfer with plasmids, transposons, and phages [7,8]. The plasmid is also invasive in the alimentary tract through adhesive receptors [3,9]. Control and management of the systemic infection appear to be dependent on the expression of T-helper 1(Th1)-type cytokine interferon- γ (IFN- γ) and clearance of bacteria from the spleen and liver is predicated on T-cell proliferative activity [10]. The association of T lymphocytes in response to Salmonella clearance is orchestrated by promoting different events such as production of pro-inflammatory cytokines like IL-1 and TNF-α, synthesis and secretion of cytotoxic secretory products, regulation of local and systemic immune response, activation of macrophages and clearance of intracellular bacteria [11,12]. Stimulating immunological T cells memory through immunization with live vaccines is important for the fast maturation and expression of CD44+ T cell response in case of challenge by pathogenic strains [13]. Immunoglobulin G (IgG) is the main effector cell of humoral immunity in the host extracellular fluids including blood, lymph and saliva. It is the most powerful immunoglobulin of systemic humoral immunity, since it is the isotype most commonly found in the circulation and tissues. The participation of the humoral immunity in opsonization and clearance of Salmonella infection also involve the IgA especially in mucosal immunity [10]. The mutagenesis of the virulent strain was achieved by tagging

with transposon Tn3. This was facilitated by using a temperature-sensitive transposon donor plasmid. The donor plasmid was introduced into *S. gallinarum* by transformation (i.e the donor plasmid fused with the target plasmid). The insertion of the transposon into the large plasmid was verified by plasmid analysis [3]. The mutant product is a rough strain (to prevent production of serum agglutinins) and it is also resistant to nalidixic acid (to facilitate the maintenance of purity). Because of the economic importance of fowl typhoid, the feasibility of parenteral vaccination of layer birds with this nalidixic acid resistant 85-kb plasmid *Salmonella gallinarum* cured with sodium dodecyl sulfate was investigated to control the disease [14]. However, there is still a need to continue the proven control measures by using more effective vaccine managements and hygiene regimens along the production chain to increase the resistance of chickens against *Salmonella sp* [15].

2. MATERIALS AND METHODS

2.1 Animals: Thirty-day-old Lohmann layer birds were divided into three groups were used in this study. They were provided with water *ad libitum* and antibiotic-free feed from one-day-old to the end of the experiment. All handling of birds and experiments were conducted following the guidelines stipulated by University of Nigeria Research Ethics Committee on animal handling and use (FMV-UNN-IACUC-2020-095). Nalidixic acid resistant 85-kb plasmid *Salmonella gallinarum* was kindly donated by Professor Paul Barrow, University of Nottingham, United Kingdom.

2.2 **Plasmid curing:** Plasmid curing of the 85-kb plasmid from the *S. gallinarum* strain: The 85-kb serovar Gallinarum nalidixic acid resistant plasmid was cured by incubating the broth culture with 5% sodium dodecyl sulphate for 18 h at 37 ^{o}C. Extraction of plasmid DNA was done using standard methods[16]. Electrophoresis of plasmid DNA was carried out with 0.8% agarose gel electrophoresis. Presence of plasmid before curing and total elimination of plasmid after curing were both identified and determined on agarose gel electrophoresis.

2.3 Serology: Thirty day-old layer birds divided into three groups of ten each were used for the experiment. At four and seven weeks of age respectively, the commercial group (SG9R) was given subcutaneously 0.2 ml containing $5x10^7$ cfu vaccine strain/bird of the fowl typhoid vaccine, the sodium dodecyl sulfate- cured nalidixic acid resistant 85kb plasmid *Salmonella gallinarum* (NAR) was given intramuscularly 0.5 ml ($5x10^9$ colony forming unit (cfu) of vaccine strain/bird while negative group served as the unvaccinated group. Blood was collected from the jugular vein and serum separated into tubes. To assess the antibody response, blood was collected from the jugular vein six days after each vaccination and serum separated. A commercial ELISA kit (Biochek, Netherlands) was used according to the manufacturer's instructions to obtain optical density (OD) values.

$$\frac{\text{OD sample} - \text{OD standard negative}}{\text{OD standard positive control} - \text{OD standard negative control}} \quad \ldots\ldots\text{Eqn 1}$$

2.4 Challenge: At ten weeks of age, all the birds were challenged orally with 1 ml of 1.2×10^9 cfu/ml of the parent Sodium dodecyl sulfate- cured nalidixic acid resistant 85-kb plasmid *Salmonella gallinarum*. The birds were observed for 5 weeks for clinical signs of morbidity and mortality.

2.5 Statistical analysis: All data were expressed as the mean value SD. Sigma plot 11.0 was used to determine the significant differences applying a Kruskal-Wallis one-way analysis of variance (ANOVA) test.

3. RESULTS AND DISCUSSION

3.1 Plasmid curing

The plasmid profile in Figure 1 showed the presence of plasmid (U_2) before curing with sodium dodecyl sulfate and absence of the plasmid (U_2S) after the curing. Other profiles are plasmids from other experimental strains. Curing of *S. gallinarum* strain 9 was necessary to reduce the risk of reversion to the virulent state in the NAR group, evidenced by an absence of morbidity or mortality after the first or booster vaccination. Elimination of this large plasmid through curing rendered the organism avirulent ajudged by the lack of morbidity during and after vaccinations. This is comparable with the

results of the experiments carried out by Barrow et al., 1987 where the 85-kb plasmid was cured using heat treatment (47 ^{o}C) for 18 h. The experiment carried out by Barrow et al showed that elimination of the large plasmid produced a strain which when inoculated orally produced no mortality but reintroduction of the large plasmid again fully restored virulence. It showed that SDS curing was safe and displayed no adverse effects in vaccinated chickens. Loss of complete or a fragment of the plasmid can be demonstrated by vertical or horizontal agarose gel electrophoresis of cleared cell lysates, or caesium chloride-ethidium bromide gradients. Plasmids can also be integrated into the bacterial host chromosome so absence of a covalently closed circular molecule does not mean that the bacteria are not plasmid-encoded [17]. The intramuscular vaccination of the NAR stimulated a systemic response of IgY as evidenced by ELISA.

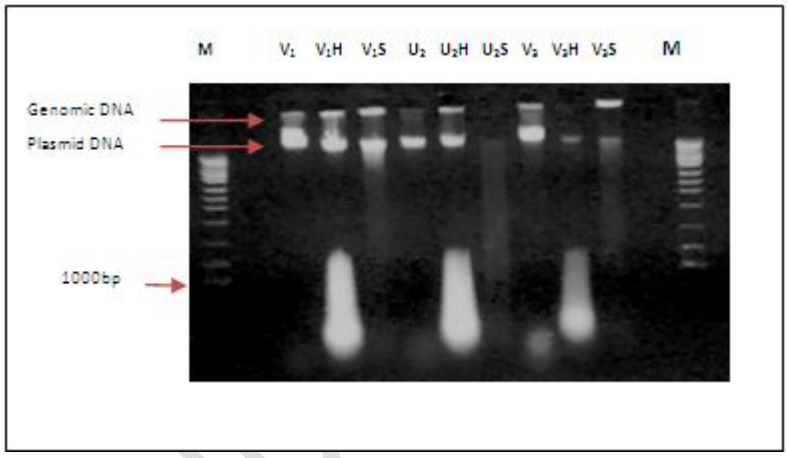

Figure 1. Plasmid profile of *S. gallinarum* strains

Key: Lane M; 1kb molecular weight marker; Lane U_2: *Salmonella gallinarum* strain with 85-kb from UK, Lane U_2 S: *Salmonella gallinarum* strain with 85-kb plasmid cured by SDS

3.2 Antibody Levels in Serum

The result in Table 1 is evidence that serum agglutinins specific to the antigen were present in NAR group irrespective of the curing**.** Primary vaccination showed IgG levels

which were low in all the vaccinated groups. The booster vaccination elicited higher immune responses above the primary vaccination. The plasmid cured group had a higher immune response above the commercial group. It is surprising that higher immune response in layer chickens was produced by a strain of *S. gallinarum* cured of its plasmid. Unfortunately, excessive antimicrobial therapy is being used to treat fowl typhoid causing an increase in the incidence of mutational antimicrobial resistance in poultry. Vaccination efficacy is also predicated on biosecurity measures, sanitation and hygienic conditions. Vaccination is necessary for protection against field strains of *Salmonella gallinarum*. The birds were vaccinated at 4 weeks old which is the age when poultry is most susceptible to fowl typhoid. The exposure of the vaccinated birds to the first vaccination resulted in the development of an immune response that took a week to provide detectable levels of antibody, and the booster dose led to a higher titre of IgG. Results of ELISA showed E-values of 0.114 ± 0.05; 0.173 ± 0.06; 0.144 ± 0.06 for NEG, SG9R, and NAR respectively after primary vaccination and E-values of 0 ± 0.00; 0.16 ± 0.2; and 0.613 ± 0.4 after booster vaccination for NEG, SG9R, and NAR respectively with SG9R showing higher IgG levels after primary vaccination and NAR after booster vaccination respectively. The higher immune responses recorded from NAR after booster vaccination is an indication of the efficacy of the developed vaccine in inducing humoral antibodies. The differences in the median values among the treatment groups WERE not great enough to exclude the possibility that the difference is due to random sampling variability; there is not a statistically significant difference (P = 0.800)

Table 1. Mean E-values of Immunoglobulin G in serum

	NEG	SG9R	NAR
Primary vaccination	0.114	0.173	0.144
Secondary vaccination	0.261	0.141	0.613

3.3 Challenge and Protection of birds

The birds in the SG9R group did not die from the virulent *S.* Gallinarum strain 9, though they looked depressed. Considerable protection was afforded by the plasmid-cured group which was lower than the mortality (48 %) recorded by Barrow, 1990. The NAR group lost three birds group as shown in Table 2 which was significantly lower than the

non-immunized birds (100 % mortality). It could be said that the degree of protection induced by the NAR was less than that induced by SG9R under experimental conditions but not statistically significant. There were signs of depression and reduced appetite in both vaccinated groups but the birds recovered. The birds in SG9R group showed some clinical signs of infection like whitish and watery faeces. After the challenge, the birds in the unvaccinated group showed serious signs of infection and morbidity leading to 100 % loss. The three birds that died from the NAR group may have died due to inappropriate or insufficient vaccine application resulting in low IgG levels and not necessarily from inadequate immune responses. The survival of all the birds in SG9R group may possibly be as a result of sufficient vaccine application protecting all the birds against the virulent 85-kb plasmid strain of *Salmonella gallinarum*. The mean antibody responses after booster vaccination in NAR group show considerable robust immunity. This robust immunity can be supported by the high oral dose (10^9) of the challenge virulent strain as compared to the 10^8 given in most challenges with virulent *Salmonella gallinarum* [10,18]. Probably, a lower dose may have given complete protection. The survival of more than 50 % of the vaccinated birds at this high dose of the challenge also implies induction of cellular immune responses though this will be investigated. This experiment shows that chickens can be protected against fowl typhoid by i.m immunization with 85-kb virulence plasmid-cured derivative of nalidixic acid resistant mutant *S. gallinarum*. The 100 % mortality in the unvaccinated is due primarily to unprotecting of the birds from the 85-kb plasmid in the challenge strain [3]. There is also an association between virulence in young and adult poultry with this plasmid-linked *Salmonella gallinarum* in earlier published works[3,19]. The plasmid was visualized by electrophoresis of using 0.7 % agarose gel and the size was estimated by direct comparison with a known plasmid [3]. Elimination of this large plasmid through curing rendered the organism avirulent ajudged by the lack of morbidity during and after vaccinations[19]. This experiment is also an investigation on the considerable protection of a cured 85-kb plasmid derivative of a strain of *Salmonella gallinarum* from the virulent parent strain and its possible use for vaccination purposes. The mechanism of protection from the 85-kb plasmid needs to be fully characterized.

Table 2. Percentage mortality of the birds after challenge with 85-kb virulent plasmid *S. enterica* serovar Gallinarum strain 9

Treatment groups	No. of birds	No. of dead birds	Percent (%) mortality
Unvaccinated group (NEG)	10	10	100
Live 9R vaccine group (SG9R)	10	0	0
Nalidixic acid resistant plasmid cured vaccine group (NAR)	10	3	30

4. CONCLUSION

The vaccination protocols generated immune responses in the birds and gave 70 -100 % protection against the virulent 85-kb plasmid *Salmonella* Gallinarum strain 9. The protective ability of the plasmid-cured *Salmonella gallinarum* was probably based on its ability to induce polyclonal active B cells. Further studies are necessary to ascertain their contributions to cellular immunity.

ETHICAL APPROVAL

As per international standard or university standard, written approval of Ethics committee has been collected and preserved by the authors.

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