

Short communication

Sodium dodecyl sulfate- cured nalidixic acid resistant 85-kb plasmid *Salmonella gallinarum* immunogenicity 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, within one (1) year.

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 ELISA. The scalability of the SDS-cured nalidixic acid resistant 85kb plasmid *Salmonella gallinarum* immunogenicity 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, immunogenicity

1. INTRODUCTION

S. gallinarum produces acute septicaemic 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 appears 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 largest and 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* [15].

2. MATERIALS AND METHODS

2.1 Animals: Thirty-day-old Lohmann layer chicks 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 bird handling 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 °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 5×10^7 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 (5×10^9 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.

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.

3. RESULTS AND DISCUSSION

3.1 Plasmid curing

The plasmid profile in Figure 1 showed the presence of plasmid (U₂) before curing with sodium dodecyl sulfate and absence of the plasmid (U₂S) 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 adjudged by the lack of morbidity during and after vaccinations. 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. Some authors reported that cells containing plasmid-specific pili on their cell surface are more sensitive to SDS [17]. The intramuscular vaccination of the NAR stimulated a systemic response of IgY.

3.2 ELISA

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.

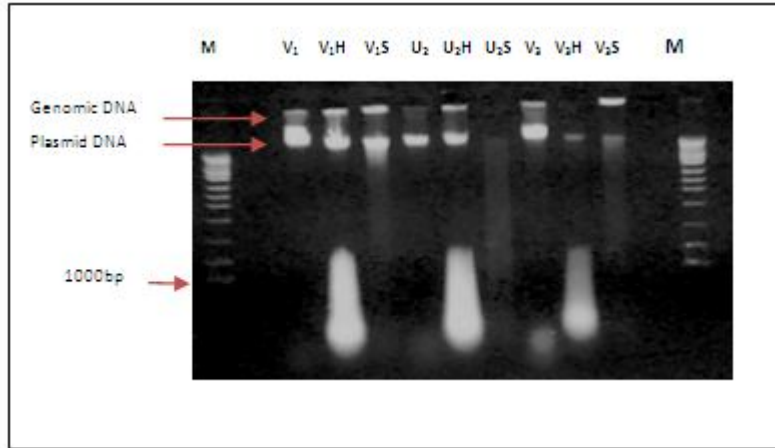


Figure 1. **Plasmid profile of *S. Gallinarum* strains**

Key: Lane M; 1kb molecular weight marker; Lane U₂: *Salmonella gallinarum* strain with 85-kb from UK, Lane U₂ S: *Salmonella gallinarum* strain with 85-kb plasmid cured by SDS

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. The NAR group lost three birds group as shown in Table1. It could be said that the degree of protection induced by the NAR was less than that induced by SG9R under experimental conditions. 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 unprotection of the birds from the 85-kb plasmid in the challenge strain [3]. There is also an association of 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 adjudged 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 1. 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 (9R)	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 polyclonally activate B cells. Further studies would be done 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.

REFERENCES

1. Shivaprasad H. Fowl typhoid and Pullorum disease. Reviews and Scientific Techniques (OIE). 2000; 19(2): 405–424.
2. Barrow PA and Freitas-Neto OC. Pullorum disease and fowl typhoid-new thoughts on old diseases: a review. Avian Pathology. 2011; 40(1): 1-13.
3. Barrow PA, Simpson JM, Lovell MA and Binns MA. Contribution of *Salmonella gallinarum* Large Plasmid toward Virulence in Fowl Typhoid. Infection and Immunity. 1987; 55(2): 388-392.
4. Jones MA, Wigley P, Page KL, Hulme SD, Barrow PA. 2001. *Salmonella enterica* serovar Gallinarum requires the Salmonella pathogenicity Island 2 Type III secretion system but not the *Salmonella* pathogenicity Island I Type III secretion system for virulence in chickens. Infection and Immunity. 2001; 69(9): 5471-5476.
5. Christensen JP, Skov MN, Hinz KH and Bisgaard M. *Salmonella enterica* serovar Gallinarum biovar Gallinarum in layers; epidemiological investigations of a recent outbreak in Denmark. Avian Pathology. 1994; 23: 489-501.
6. Barrow PA and Lovell MA. The association between a large molecular mass plasmid and virulence in a strain of *Salmonella pullorum*. Journal of General Microbiology. 1998; 134(8): 2307-2316.

7. Foley SL, Johnson TJ, Ricke SC, Nayak R, Danzeisen J. Salmonella pathogenicity and host adaptation in chicken-associated serovars. *Microbiology and Molecular Biology Reviews*. 2013; 73(4): 582-607.
8. Shah DH, Lee M, Park J, Lee J, Eo S, Kwon J, Chae J. Identification of *Salmonella gallinarum* virulence genes in a chicken infection model using PCR-based signature-tagged mutagenesis. *Microbiology*. 2005;151: 3957-3968.
9. Singh V, Singh VP, Gupta PK, Chaudhuri P. Plasmid profile and virulence analysis of *Salmonella Gallinarum* Indian Isolates. *Journal of Applied Animal Research*. 1996; 9: 129-133.
10. Filho CP, Moura BS, de Almeida AM, Montassiera HJ, Barrow PA, Berchieri A. Aspects of humoral and cellular immune response generated by different vaccine programs before and after *Salmonella enteritidis* challenge in chickens. *Vaccine*. 2012; 30(52): 7637–7643.
11. Delgoffe GM and Powell JD. Feeding an army: The metabolism of T cells in activation, anergy, and exhaustion. *Molecular Immunology*. 2015; 68: 492-496.
12. Dougan G, John V, Palmer S, Mastroeni P. Immunity to Salmonellosis. *Immunology*. 2011; 240(1):196–210.
13. Holmkvist P, Roepstorff, Uronen-Hansson K, Sanden C. A major population of mucosal memory CD4+ T cells, coexpressing IL-18R α and DR3, display innate lymphocyte functionality. *Mucosal Immunology*. 2015; 8(3): 545-558.
14. El-Mansi M, Anderson KJ, Inche CA, Knowles LK, Platt DJ. Isolation and curing of the *Klebsiella pneumoniae* large indigenous plasmid using sodium dodecyl sulphate. *Research in Microbiology*. 2000;151: 201–208.
15. European Food Safety Authority, European Centre for Disease Prevention and Control. Report on trends and sources of zoonoses, zoonotic agents and food-borne outbreaks in 2016. EFSA. 2017; 15(12):5077.
16. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. *Journal of Bacteriology*. 1981; 145:1365-1373.
17. Trevors JT. Plasmid Curing in bacteria. *FEMS Microbiology Letters*. 1986; 32(3-4):149-157.

18. Wigley P. Genetic resistance to Salmonella infection in domestic animals. Research in Veterinary Science. 2004; 76:165-169.

19. Barrow, P.A. 1990. Immunity to Experimental Fowl Typhoid in Chickens Induced by a virulence Plasmid-Cured Derivative of *Salmonella gallinarum*. Infect. Immun. 58(7): 2283-2288

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