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Full Length Research Paper

Production of high polyvalent antisera against *Salmonella*

C. C. Ibebuike[1], S. C. Yah[2]*, and N. O. Eghafona[1]

[1]Department of Microbiology, Faculty of Life Sciences, University of Benin, Benin City, Edo State, Nigeria.
[2]Department of Biological Sciences, College of Science and Technology, Covenant University, Km 10 Idiroko Road, P. M. B 1023 Ota, Ogun State, Nigeria.

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The production of polyvalent *Salmonella* antisera was carried out by immunizing rabbits. The antigens were obtained from four strains of *Salmonella* species. The production of the antisera was achieved by administration of antigen emulsions in alum throughout the duration of immunization, antigen emulsions in alum used only at the primary immunization while subsequent antigens were administered intravenously without alum throughout the duration of the immunization period. The results obtained showed high titres of *Salmonella* antisera that ranged from 1:1024 – 1:4096, 1:256 – 1:1024 and 1:64 – 1:256 for antigen emulsions in alum throughout the immunization period, antigen emulsions in alum for primary immunization only and antigen without alum, respectively. Specificity test revealed that the antisera produced were specific to *Salmonella* antigen although slight reactions were observed with *Escherichia coli* at a very low titre ranging from 1:4 – 1:16. The avidity test showed that the antisera produced were of high standard and ranged from 12 - 32 s. There was a significant difference (P < 0.05) between the commercially produced antigen and locally produced antigen against the antisera. The work therefore, demonstrated the possibility of producing high *Salmonella* antisera by immunizing rabbits with *Salmonella* antigens.

Key words: *Salmonella* O and H antigens, antisera production.

INTRODUCTION

In recent years, the production of antibodies in laboratory animals has become an essential part of many research projects. The production of these antibodies to specific and nonspecific antigens is a tool utilized in nearly all fields of biomedical research (Robson, 1995). The increased occurrence of *Salmonella* infections in recent years have accentuated the need for the production of *Salmonella* antisera, which is used for serotyping of *Salmonella* as a base for proper diagnosis, identification of sources of infections and control of food products (Aliexo et al., 1984; Kimmi et al., 2008). *Salmonella* are Gram-negative, flagellated, facultative anaerobic that causes salmonellosis which is a self-limiting food poisoning (gastroenteritis), but occasionally manifests as a serious systemic infection (enteric fever) which requires prompt antibiotic treatment (Oldfield, 2002). The bacilli posses three major antigens: H (flagella antigen), O (somatic antigen) and Vi (virulence antigen) possessed by only a few serovars (Duguid et al., 1989 and Rubin, 2004). The H antigen may occur in either or both forms called phase 1 and 2. The organism tends to change from one phase to another (Deng, 2003). O antigens occur on the surface of the outer membrane and are determined by specific sequences on the cell surface (Kondoh and Hotam, 1994). Vi antigen is a superficial antigen overlying the O antigen; it is present in a few serovars, the most important being *Salmonella typhi* (Giannella, 1996; John et al., 2001).

Antigenic analyses of *Salmonella* by using specific antisera offer clinical and epidemiological advantages. Determinations of antigenic structures permit one to identify the organisms chemically and assign them to serogroups each containing many serovars (Giannella, 1996). The antigens also provide a useful epidemiologic tool which could be used to determine the source of in-

*Corresponding author. E-mail: yahclar@covenantuniversity.com, becckskop@yahoo.com.

fection and its mode of spread. In order to raise an antiserum to a particular antigen, the first requirement is the need for a reliable supply of antigen. The purity of the antigen that is injected into an animal is very important. If the antigen is impure, the resulting antisera may recognize the impurities with equal or greater affinity (Kerridge, 1962; Nossal et al., 1964). The production of antisera with high titres and specificity to *Salmonella* is therefore of high interest. This is because of the need to develop rapid immunoassay procedures for the detection of *Salmonella* in food and clinical specimens. The aim of this work is to produce high titre polyvalent antisera using different methods: by administration of antigen emulsions in alum throughout the duration of immunization, antigen emulsions in alum used only at the primary immunization and antigen injected without alum. This will enable the test for the specificity and avidity of the antisera produced as well as compare with standard antigen on the antisera produced.

METHOD

Organisms

The organisms used were obtained from the Nigeria Institute for Medical Research (NIMR) Lagos. They include two strains of *S. typhi* (B015 and B026) and four strains of *Salmonella enteritidis* (B003, B006, B013 and B028).

To identify and purify the *Salmonella* isolates, they were cultured into selenite F broth in screw-capped bottles and incubated at 37°C for 3 - 5 days. Subcultures were then made into plates of deoxychocolate citrate agar, and incubated for another 24 h. Colonies with black centers were sub-cultured onto nutrient agar and incubated for 16 - 18 h to obtain cells at logarithm phase. The cultures on nutrient agar plates were subjected to Gram-staining, motility, urease production, hydrogen sulfide production and citrate utilization tests. All Gram-negative, rod-shaped, motile, urease-negative isolates that produced on alkaline slant with acid butt on triple sugar Iron agar were regarded as species of the genus *Salmonella* according to Cowan and Steel (1993).

Animals

Forty two female white rabbits from the same breed (2.0 to 2.5 kg) used for the experiment were obtained from the Department of Microbiology, University of Benin. They were arranged into seven (7) groups of two rabbits each with one group serving as the control. Group one was immunized with O antigen mixed with adjuvant while group two was immunized with O antigen without adjuvant. Groups three and four were immunized with H antigen with adjuvant and H antigen without adjuvant respectively. Group five were immunized with O antigen with adjuvant only at the initial immunization, while group six were immunized with H antigen and adjuvant only at the initial immunization. Subsequent immunizations for these groups were without adjuvant. The experiment was carried out in triplicate to have a good significant value. The animals were housed in Iron steel cages (1 rabbit per cage) with 12 h light and dark periods. The animals were certified by Johvet Vet Clinic and performed with approval of the animal care and use committee in Nigeria.

Preparation of H antigen

The organisms (salmonellae) were picked with the aid of a sterile wire loop from the stock culture and streaked into nutrient agar plates and incubated at 37°C for 16 - 18 h to obtain cells at logarithm phase. The organisms were inoculated into 6 tubes of 5 ml of sterile normal saline and matched with McFarland's standard. The cells were then incubated at 100°C for 30 min according to Duguid et al. (1989). After incubation, the tubes were centrifuged at 10,000 g for 15 min. The supernatants were then pooled together where 5 ml was then added to 5 ml of alum. 2 ml of antigen emulsions in alum was injected into each rabbit (Groups three and six) at different sites (upper and lower limbs) in 0.5 ml per site (George et al., 1985). Also rabbits (Group four) were injected with 2 ml each of H antigen without alum through the marginal ear vein.

Preparation of O antigen

From the stock culture, the organisms were streaked into nutrient agar plates using sterile wire loop and incubated at 37°C for 16 - 18 h to obtain cells at logarithm phase. The cultures were then inoculated into six test tubes (each in one test tube) containing 2 ml of nutrient broth. Equal volumes of 96% ethanol was added and incubated at 37°C for 4 h (Duguid et al., 1989). After incubation, they were then centrifuged at 10,000 g and the supernatants decanted. The deposits were washed by suspending in sterile normal saline and re-centrifuged. The supernatants were again decanted and the deposits re-suspended in 5 ml of normal saline. The O antigens were then pooled together into a sterile container. 5 ml of these antigens were poured into a sterile container and equal volume of alum was added and properly mixed. The antigen emulsion in alum as well as antigen only was also administered according to George et al. (1985).

Immunization

The rabbits were immunized according to the immunization schedule of Rockland's custom antibody production service for the production of antisera. Alum was used in place of Freund's adjuvant. The antigen was administered subcutaneously at multiple sites at the upper and lower limbs when given with adjuvant and injected intravenously through the marginal ear vein without adjuvant. The immunization schedule was conducted as follows: Pre bleeding, day 1 initial immunization, day 7 boosters, day 14 boosters, day 28 boosters, day 38 booster and 45 terminal bleed.

The ear of the rabbits was swabbed with alcohol and then with cotton wool soaked in Xylene. Sterile syringe (2 ml) and needle (25G) was used for the collection of blood through the marginal ear vein. Terminal bleeding was done by cardiac puncture. The rabbit was anesthetized with an intramuscular injection of Ketamine hydrochloride 10 mg/ml (Kamala Overseas, Marine Drive Mumbai) and supported with localized nerve block injection using lidnocaine 2% (Kamala Overseas, Marine Drive Mumbai). It was then clipped on a dissecting board and sterile syringe (10 ml) and needle (21G) was used to collect the blood from the heart. The blood was then centrifuged at 10000 g and the serum poured into sterile containers.

Agglutination test

The tube agglutination was done with normal saline using four fold serial dilutions while the slide agglutination test was done using the standard methods. The widal kit (Cromatis, Spain) was used as the standard.

Specificity test

Some members of the enterobacteriaceae family have common antigenic determinants and as such, *Proteus*, *Escherichia coli* and

Table 1. Agglutination titre of test H and standard H antigens obtained when adjuvant 1024 was used throughout the period of immunization.

Organism	Ref. no	Test H titre	Standard H	Titre
S. typhi	BO_{15}	1:4096	*S. paratyphi* H	1:64
S typhi	BO_{26}	1:4096	*S. paratyphi* AH	1:256
S. enteritidis	BOO_3	1:4096	*S. paratyphi* BH	1:256
S. enteritidis	BOO_6	1:	*S. paratyphi* CH	1:256
S. enteritidis	BO_{28}	1:4096		
S. enteritidis	BO_{13}	1:4096		

Table 2. Agglutination titre of test O and standard O antigens obtained when adjuvant was used throughout the period of immunization.

Organism	Ref. no	Test O titre	Standard O	Titre
S. typhi	BO_{15}	1:4096	*S. paratyphi* H	1:256
S typhi	BO_{26}	1:4096	*S. paratyphi* C	1:64
S. enteritidis	BOO_3	1:1024	*S. paratyphi* AH	1:256
S. enteritidis	BOO_6	1:4096	*S. paratyphi* BH	1:256
S. enteritidis	BO_{28}	1:4096		
S. enteritidis	BO_{13}	1:1024		

Klebsiella species were used to test for the specificity test with the *Salmonella* antisera produced. The slide agglutination and tube agglutination methods were used as previously described.

Avidity test

This was used to determine the time taken for agglutination to occur. The slide agglutination method was used.

RESULTS AND DISCUSSION

A total of 42 rabbits were used for the production of *Salmonella* antisera. The antisera titres produced varied slightly among the various methods employed. The results of immunizing *Salmonella* with adjuvant throughout the immunization period, showed agglutination titres for both test H antigen and standard H antigen which ranged from 1: 1024 to 1:4096 and 1: 64 to 1:256, respectively (Table 1). The agglutination titre obtained for test O antigen and standard O antigen ranged from 1:1024 to 1:4096 and 1:16 to 1:256, respectively (Table 2).

The immunization of rabbits with *Salmonella* in adjuvant (used only for primary immunization), produced agglutination titre ranged of 1:256 to 1:1024 for both H and O antigens respectively (Table 3). The results of immunizing *Salmonella* antigens without adjuvant produced agglutination titre which ranged from 1:64 to 1:256 (Table 4). The antigen titre produced when compared with the standard antigen showed a significant difference ($P < 0.05$) between the two.

Table 3. Agglutination titre obtained when adjuvant was used only at the primary immunization.

Organism	Ref. no	H Titre	O Titre
S typhi	BO_{26}	1:1024	1:1024
S. enteritidis	BOO_3	1:1024	1:1024
S. enteritidis	BOO_6	1:1024	1:256
S. typhi	BO_{15}	1:1024	1:1024
S. enteritidis	BO_{28}	1:256	1:256
S. enteritidis	BO_{13}	1:256	1:1024

Table 4. Agglutination titre obtained when rabbits were immunized without adjuvant.

Organism	Ref .no	H Titre	O Titre
S typhi	BO_{26}	256	256
S. enteritidis	BOO_3	256	256
S. enteritidis	BOO_6	256	256
S. typhi	BO_{15}	256	256
S. enteritidis	BO_{28}	64	256
S. enteritidis	BO_{13}	256	256

The specificity test showed a positive *E. coli* tube agglutination titre that ranged of 1:4 – 1: 16 for both H and O antisera. The result of the avidity test ranged from 12 – 31 s for both H and O antisera.

Problems associated with identification of *Salmonella* in both clinical and research purposes were the major reason for the undertaking of this work. The use of adequate

isolation procedures and differential biochemical tests is of primary importance. However, complete serological characterization of *Salmonella* is required for successful detection of the pathogen (Aliexo et al., 2001).

According to George et al. (1985), experimental and diagnostic antisera can be prepared in the laboratory by immunizing rabbits with the appropriate antigens. In this research, immunization of rabbits with immunogenic H and O *Salmonella* antigens resulted in high yield of antisera. This was in agreement with the work of Johnstone (1989), where he reported that *Salmonella* antigens can be stereotyped with confidence after appropriate dilution of the antisera. The result obtained compared favorably with earlier studies of Fey et al. (1975) and George et al. (1985) in which higher antisera titre were obtained.

The antigen emulsions used by Vaitukaitis et al. (1971) for the production of antisera for hormone haptens contained a final concentration of 2.5 mg of heat-killed *Mycobacterium tuberculosis* per ml and each animal was administered antigen emulsion as well as 0.5 ml of crude *Bordetella pertussis* vaccine, presumably to enhance the immune response to antigens. Also, in the work done by George et al. (1985) the final concentration of heat-killed *M. tuberculosis* in antigen emulsion used for primary immunization was only 0.3 mg/ml and the use of *B pertussis* vaccine was omitted. This is because *Salmonella* flagellins are known to be highly immunogenic (Ada et al., 1963; Nossal et al., 1964 and Richman, 2004) and the unnecessary administration of bacterial antigens other than *Salmonella* flagellins to the rabbits are undesirable. From the results obtained, it shows that use of adjuvant throughout immunization period yielded better result (titre ranged from 1:1024 – 1:4096) than when adjuvant was not used (1:64 – 1:256). However, the result obtained when adjuvant was used only at the initial immunization gave high titre compared with the immunization without adjuvant. The tire (1:256 – 1:1024) could not be sustained because of the absence of adjuvants. This could be attributed to the fact that adjuvants are known to enhance the efficiency of immunogenic substances and as such could have stimulated the production of more antisera in the rabbits (Broderson, 1989).

The titres produced for both H and O antigens in this work were exceptionally high especially the adjuvant coupled antigens as compared to commercially available antisera that usually ranged from 1:20 to 1:320 and above. According to earlier reports by George et al. (1985) this could be due to the different methodologies used for antisera production and consequently much lower titres of antibodies in commercial production.

The antigen titre produced when compared with the standard antigen showed a significant difference ($P < 0.05$) between the two. The low titre observed in the standard antigen can be attributed to some factors such as shelf life as well as storage. The result of specificity test showed that only *E. coli* reacted with the *Salmonella* antisera and the titre level ranged from 0 – 1:16. Dilution of sera above a titre of 1:16 resulted in a negative agglutination result as no visible reaction occurred.

The result of avidity test indicated that the antisera produced were of good quality. These were comparable to those earlier reported by Hansen et al. (2006) where they found that immunoglobulin (IgG) avidity differentiated between acute and persistent infection with *Salmonella* Dublin in cattle.

From the results obtained, use of antigen in adjuvant emulsion for immunization could be good for the generation of high antisera titre over a long period of time. Although immunization without adjuvant yielded antisera it was observed that the high titre level could not be maintained for a long period due a decline in antigen supply.

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