Universal enzyme-linked immunosorbent assays (ELISA) and utility in the immunodiagnosis of Salmonellosis.

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Abstract:

The aims of this research are confirming the feasibility of hybrid immunoglobulin-binding reagents, its used in ELISAs for IgG/IgY detection and detecting specific antibodies against an infectious microorganism (*Salmonella* spp) in various animal species using a universal diagnostic ELISA. Hybrid immunoglobulin-binding bacterial proteins (IBP) including recombinant protein LA, recombinant protein LG, and recombinant protein AG have been produced for improvement of their binding affinity to a much larger number of immunoglobulins. This hybrid bacterial protein thus represents a powerful tool for binding, detection, and purification of immunoglobulins and their fragments. However, SpLA-LG-peroxidase and SpLAG-anti-IgY-peroxidase were produced by the periodate method. They have shown to be effective reagents. Their binding affinity to immunoglobulins surpasses previous hybrid IgG-binding proteins reported, including the most known SpAG, SpLA and SpLG. The IgY fraction was isolated from the egg yolks of a variety of birds including species of chicken, bantam hen, guinea hen, quail, goose, duck, wild and domestic pigeon, parakeet, cattle egret, pheasant, and ostrich. The IgY fraction was isolated by the chloroform-polyethylene glycol (PEG) method. An ELISA for anti-*Salmonella* spp antibodies was employed with some modifications to determine the presence of antibodies in humans, laying hens, geese, quails, and pigeons. *Salmonella* are motile, flagellated rod-shaped zoonotic pathogens which may survive with or without oxygen. They belong to the family Enterobacteriaceae and is implicated with typhoid fever and food-borne illnesses. This pathogen is associated with several diseases, which may become fatal and negatively impact the health of individuals and various economies globally. The poultry industry is most impacted and vulnerable to the onslaught of this pernicious microbe.

Keywords: Immunoglobulin-binding bacterial protein (IBP), *Salmonella* spp., epidemiological survey.

Introduction

Hybrid immunoglobulin-binding bacterial proteins (IBP) including recombinant protein LA [1], recombinant protein LG [2] and recombinant protein AG [3] has been produced for the improvement of their binding affinity to a much larger number of immunoglobulins. In addition, they have been used in the diagnosis of zoonosis infections in tens of zoo animals worldwide [4]. This hybrid bacterial protein thus represents a powerful tool for binding, detection, and purification of immunoglobulins and their fragments.

By chemical protein conjugation we engineered various hybrid bacterial Ig receptors with the capacity to bind to many immunoglobulins from mammalian and avian species. It includes SpLA-LG-peroxidase and SpLAG-anti-IgY-peroxidase produced by the periodate method [5].These molecules display a very low background in immunoassays. It makes them feasible as conjugates or coated to the solid phase of ELISAs for the detection of specific antibodies [4,6].

In previous work the binding affinities of individuals immunoglobulin-binding reagents were tested, and researchers realized that none of the individual proteins including protein-A, G and L bind extensively. None of this Ig-binding reagent bind with a good binding affinity to immunoglobulins. However, their fusions including protein AG (SpAG), protein LG SpLG) and protein LA (SpLA) were more effective [7]. Figure 1 shows the SDS-PAGE, which depicts the creation of fusion proteins to detect antibodies. Several hybrid proteins can be displayed in the protein electrophoresis, which have been extensively studied [8].

Salmonella are motile, flagellated rod-shaped Zoonotic pathogens which may survive with or without oxygen and do not absorb crystal violet stain. They are decolorized by alcohol due to their outer lipopolysaccharide membrane and thin peptidoglycan layer. They belong to the family Enterobacteriaceae and is implicated with typhoid fever and food-borne illnesses. This pathogen is associated with intestinal disease which may become fatal and has negatively impact the health of individuals and various economies globally. The poultry industry is most impacted and vulnerable to the onslaught of this pernicious microbe. The Lipopolysaccharide Somatic O antigen, flagellar H and virulent V antigenic structure determines the serotype designate of *Salmonella* species [9].

Salmonellosis is an inflammation of the intestinal mucosal lining by *Salmonella* bacteria infiltration resulting in painful symptoms. Both man and animal are vulnerable. Salmonellosis is caused by ingestion of *Salmonella* with food [10]. The main route of transmission of *Salmonella* microbe is fecal-oral [11]. Ingestion of small concentration of *Salmonella* such as 6 cells with contaminated food or water will lead to salmonellosis infection. *Salmonella* resists the acidic conditions imposed by gastric juice and amino acids in the stomach. Incubation may last for six to twenty-four hours [12].

The bacteria adhere itself to receptors on the epithelium membrane of the intestine where it stimulates enterotoxins resulting in inflammation. One may experience stomach cramps, vomiting, nausea, diarrhea, ulcer and an elevated body temperature. Systemic breach of the

blood vessels and entry of the bacteria into the blood stream may be fatal [12]. Salmonellosis may persist up to 4-7 days. The disease usually resolves itself and there is usually no need for antibiotics in healthy individuals although it is commonly administered especially among the elderly, immuno-compromised persons, and young children.

Salmonella Enteritidis as a global problem with the most implicated cases of salmonellosis in Asia, the Americas, Africa, and Europe. *Salmonella* Typhimurium presently leads Enteritidis as the prominent source of salmonellosis in America. *Salmonella* Enteritidis serotype is associated with many death and gastroenteritis cases in the United States during 1990 to 2001. This incurred a loss of over eight hundred million dollars to the United States. There is concern that for every twenty thousand eggs produced one will be contaminated by *Salmonella* Enteritidis considering that there is production of a little over sixty billion eggs yearly by the Industry [13].

The aims of this research are to confirm the feasibility of hybrid immunoglobulin-binding proteins used in ELISAs for the study of the binding affinity to IgG/IgY of many pet, laboratory, farm and wild animals. We aim to detect specific antibodies against an infectious microorganism (*Salmonella* spp) in various animal species using a universal ELISA.

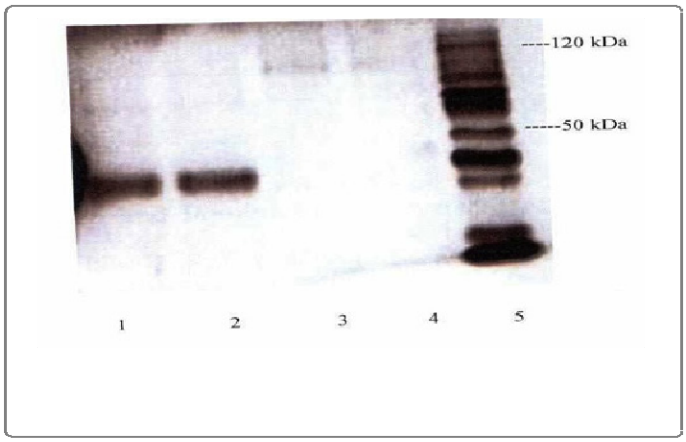
Materials and Methods

Immunoglobulins

The IgY fraction was isolated from the egg yolks of a variety of birds including species of chicken, bantam hen, guinea hen, quail, goose, duck, wild and domestic pigeon, parakeet, cattle egret, pheasant, and ostrich. The IgY fraction was isolated by the chloroform-polyethylene glycol (PEG) method (Polson method, 1990) [13,14]. Purified immunoglobulin (Ig) are purified using an antibody purification system based on Protein A-affinity chromatography (PURE-1A) supplied by Sigma-Aldrich. The manufacturer's instructions were followed as described in [15]. Other mammalian IgG used in this assay were purchase to Sigma-Aldrich or donated.

Conjugations of the immunoglobulin-binding bacterial antigens.

Figure 1and 2 show horseradish peroxidase (HRP) labelled SpA, SpG and/or SpL conjugates and their hybrid proteins as SpLAG-anti-IgY-HRP and SpLA-LG-HRP, which were prepared using the periodate method described by Nakane and Kawoi [16]. From all conjugate prepared SpLAG-anti-IgY-HRP and SpLA-LG-HRP were less polymeric than other conjugates. The optimal working dilutions of the conjugates range from 1:2000 to 1:5000 [17].



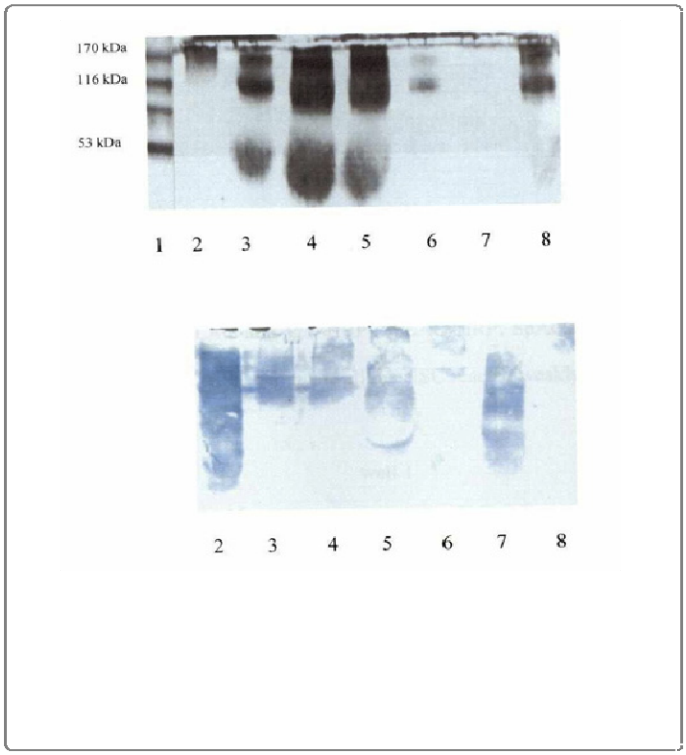


Figure 1: 10% Denaturing SDS-PAGE of SpLA-LG-HRP conjugate in lanes 1-2 about 30 kDa and SpLAG-anti-IgY-HRP conjugate in lanes 3-4 that was denatured by heat and the protein bands are very faint. Lane 5 is the molecular weight (MW) marker. **Figure 2** shows denaturing SDS-PAGE and immunoblot analysis of chimeric conjugates: lane 1 MW marker, lane 2 SpLA-HRP (Sigma). The next conjugates were homemade lane 3 SpLA-HRP, lane 4 SpLG-HRP), lane 5 SpG-HRP, lane 6 SpAG-HRP, lane 7 SpLA-LG-HRP and lane 8 SpLAG-HR. Immunoblot analysis shows that all chimeric conjugates interacted with human serum immunoglobulins and it confirmed their polymeric nature. Line 7 shows SpLA-LG-HRP a conjugate that will be used as a reagent in the development of a universal ELISA.

Specimens

A total of 139 specimens: eggs from laying hens (51), geese (10), quails (25) , and domestic pigeons (14) were investigated for the presence of anti-Salmonella antibodies. They were purchase at local grocery outlets including supermarkets situated in the corporate areas of St Andrew, in addition to local markets and a bird aviary where many eggs were collected. Egg specimens purchased were aseptically put into sterile bags. All egg samples were placed into an igloo with ice packs and further transported to the laboratory [18]. Human serum samples from patients with cervical dysplasia (30 patients) and normal healthy subject (10) were also assessed by the presence of anti-Salmonella antibody [19].

The samples in question are IgYs from duck, ostrich, parakeet, Eagle egret, Bantam hen, pheasant, chicken, domestic pigeon, wild pigeon, goose, guinea hens and quail. The mammalian IgGs, which binding interactions with bacterial proteins will be studied include antibodies from pig, human, skunk, coyote, raccoon, mouse, hamster, bovine, goat, rabbit, cat, donkey, mule, horse, dog, guinea pig and rat.

Basic ELISA protocol using SpLA-LG-HRP and SpLAG-anti-IgY-HRP.

The microplate is coated directly with the avian or mammalian immunoglobulin in carbonate-bicarbonate buffer pH 9.6 overnight. The microwell is then treated with a blocking solution and washed. After that, the bacterial protein conjugate is added and incubated for 1h. The microplate is rewashed again and added the enzyme substrate. After an incubation procedure in the dark for 15 min, the reaction is stopped and the microwell read at 450 nm. Samples which are positive are above the cut-off point, calculated from the mean of negative controls.

ELISA for anti-Salmonella antibodies in several animal species.

An enzyme-linked immunosorbent assay to detect anti-Salmonella antibodies in humans and several avian species was performed. Ninety-six well polystyrene microplates (U-shaped bottom, Sigma-Aldrich) were incubated at 4°C and overnight with 1 µg/well of the LPS from Salmonella Typhimurium. The microplates were washed and blocked with PBS-tween 20 pH:7.4 for 1 hour at room temperature (RT). The microplates were then rewashed x 4 times as previously performed. Then, a 50 µl of human serum or 50 µl of IgY sample (1mg/ml) from laying hens, geese, quails, and pigeons was added previous IgY purification by the method of Polson [13]. After incubating for one hour at RT, the microplates were washed again. Fifty (50) µl SpLAG-anti-IgY-HRP conjugate in dilutions 1:5000 with PBS-Tween-20 was added. After a further incubation and washing procedure 50µl tetramethylbenzidine (TMB) was added onto each well. Microplates were further incubated for 15 minutes in the dark, and after that 50µl of 3M HCl was added to each well to stop the reaction. The microplates were read at 450 nm in a microplate reader. The cut-off was calculated as the mean optical density value (XOD) times two. The positive and negative controls (five each) were humans with very high or no titer of anti-Salmonella antibodies, respectively [19]. The coefficient of variation intra-assay and inter-assay were also calculated as a measure of the reproducibility.

Statistical analysis

Statistical Package for the Social Sciences (SPSS) version 25 was used for the calculation of the coefficients of variation and 95% confidence intervals.

Ethical approval: This research was approved by the University of West Indies (UWI) Ethics Committee (Mona Campus) in Jamaica, West Indies.

Result and Discussion

SpLA-LG Direct ELISA

This ELISA is used to study the interaction of proteins SpLA-LG-HRP with avian and mammalian immunoglobulins. The 96 well microtiter plate is coated overnight at 4°C with 1 µg/µl per well of IgG or IgY (1mg/ml) in carbonate-bicarbonate buffer pH 9.6. Then plate is treated with bovine serum albumin solution and washed 4X with PBS-Tween. Then 50 µl of peroxidase-labeled-SpLA-LG conjugate diluted 1:2000 in PBS-non-fat milk is added to each well and incubated for 1 hour at RT. After that, the plate is washed 4X with PBS-Tween-20. Then, pipette 50 µl of 3,3',5,5' - tetramethylbenzidine (TMB; Sigma-Aldrich) onto each well. The reaction is stopped with 50 µl of 3M H2SO4 solution. The plate is assessed visually for the development of color and read in a microplate reader at 450 nm. After that, a cut-off point is calculated as the mean of the optical density of negative controls times two [15]. The cut-off point is 0.268. Chicken IgY is the negative control from which was calculated the affinity ranges.

The SpLA-LG direct ELISA is a novel immunoassay that examine the binding affinity of a novel hybrid immunoglobulin-binding protein to immunoglobulins from a variety animal species. The authors are not aware of developing an ELISA using this protein conjugate for immunodetection. The result show that SpLA-LG binds preferentially to mammalian IgG. It binds strongly to 46.7% (14 out of 30 samples). It binds moderately to 13.33% of the immunoglobulin panel (4 out of 30 samples). SpLA-LG binds weakly to 10% of Igs (3 out of 30 samples) and it does not bind to 30% of Ig panel (9 out of 30). This protein binds specifically and preferentially to mammalian IgGs. However, it is not a good immunological marker for detection of avian immunoglobulins, except few exceptions as IgY of bantam hen, ostrich, and duck. This hybrid protein was improved by the insertion of a molecule of anti-chicken IgY (Promega) in the SpLA-LG molecule, using the periodate method. Several ratio of bacterial protein-enzyme where assayed until finding the most appropriate one that was a ratio 1:1. However, by judging by the SDS-PAGE results, an amount of unconjugated peroxidase is present in the conjugate but it is eliminated with the washing steps after adding the labeled immunoglobulin-binding protein to the immunoassay.

Table 1: <u>SpLA-LG Direct ELISA</u>

Immunoglobulins	XOD results	Binding affinity	Coefficient of variation (CV) intra-assay (%)	CV inter-assay (%)
Duck	0.288	+	3.27	4.95
Ostrich	0.315	+	4.18	6.13
Parakeet	0.011	-	3.09	5.35
Eagle egret	0.090	-	3.02	4.58
Bantam hen	0.287	+	3.75	5.52
Pheasant	0.112	-	4.78	6.09
*Chicken	0.134	-	4.01	5.89
Domestic pigeon	0.089	-	2.89	4.06
Wild pigeon	0.083	-	3.11	4.78
Goose	0.108	-	4.05	8.05
Guinea hen	0.095	-	3.23	5.56
Quail	0.125	-	3.56	7.03
Rat	0.754	++	3.44	6.62
Dog	0.995	+++	4.08	7.10
Horse	0.856	+++	4.77	6.85
Mule	0.680	++	3.89	5.73
Donkey	0.814	+++	3.70	6.17
Cat	0.655	++	3.68	4.95
Rabbit	1.058	+++	4.18	7.75
Goat	0.886	+++	5.17	8.93
Bovine	0.850	+++	4.88	6.23
Mouse	1.122	+++	4.22	5.60
Raccoon	0.907	+++	4.06	6.03
Coyote	0.875	+++	3.95	5.55
Skunk	1.259	+++	4.05	6.36
Hamster	0.751	++	3.99	6.12
Guinea pig	1.004	+++	4.72	8.08
Human	1.356	+++	3.39	4.97
Pig	1.202	+++	4.06	6.94
Dolphin	1.137	+++	3.68	6.70

<u>Affinity ranges:</u>

XOD < 0.268= - (no binding)

0.268-0.535= + (weak)

0.536-0.803= ++ (moderate)

XOD > 0.804= +++ (strong)

SpLAG-anti-IgY Direct ELISA

This ELISA is used to study the interaction of proteins SpLAG-anti-IgY-HRP with avian and mammalian immunoglobulins or antibodies. The 96 well microtiter plate is coated overnight at 4°C with 1 µg/µl per well of IgG or IgY (1mg/ml) in carbonate-bicarbonate buffer pH 9.6. Then plate is treated with bovine serum albumin solution and washed 4X with PBS-Tween. Then 50 µl of peroxidase-labeled-SpLAG conjugate diluted 1:2000 in PBS-non-fat milk is added to the microplate, which is then incubated for 1 hour at RT. After that, the microplate is washed 4X with PBS-Tween-20. Then, pipette 50 µl of 3,3',5,5' - tetramethylbenzidine (TMB; Sigma-Aldrich) onto each well. The reaction is stopped with 50 µl of 3M H2SO4 solution. The microwell is assessed visually for the development of color and read in a microplate reader at 450 nm. After that, a cut-off point is calculated as the mean of the optical density of negative controls times three [15]. The higher the OD value the higher will be the binding affinity of the bacterial proteins to immunoglobulins. The cut-off point is 0.261.

The SpLAG-anti-IgY-HRP conjugate is one of the most versatile reagents for standardization of universal ELISAs. It binds strongly to 45.16% of the Ig panel (14 out of 31 samples). It also binds moderately to 25.8% of immunoglobulins including various avian and mammalian immunoglobulins as IgYs of ducks, bantam hens, ostriches, and chickens. It bound weakly to some immunoglobulin Y from avian species including parakeet, eagle egret, pheasant, both species of pigeons, goose, guinea hen and quail. This conjugate surpasses the effectivity of the previous SpLA-LG-HRP that binds partially to avian immunoglobulins. The SpLAG-anti-IgY-HRP having primary anti-chicken IgY antibodies that cross-react with all IgY of the panel gives advantages to the whole molecule, because nowadays it is the only conjugate with affinity for avian and mammalian antibodies.

The results of the binding affinity of bacterial protein to immunoglobulins in the immunoassays suggest that neither the SpLA-LG-HRP nor the SpLAG-anti-IgY-HRP present steric hindrance in their interaction with antibodies; even when SpA and SpG bind to the Fc fragment of antibodies [21,22] and protein L binds to light chains or Fab regions of many antibodies [23]. It is more advantageous using IBP than primary or secondary specific antibodies. On the other hand, ELISAs that use peroxidase labeled primary of secondary antibodies are highly specific for an animal species. For example, the detection of anti-Salmonella antibodies in humans using an specific human ELISA only allow the detection of antibodies in man; however when you use any immunoglobulin-binding protein, combined with an enzyme, the ELISA becomes an universal assay that allows the determination of specific antibodies in a greater amount of species with a higher sensitivity and specificity. In the case that is presented here, anti-Salmonella antibodies are detected in five species including a mammal (human) and four avian species more with only one standardized universal ELISA. The turtle IgY is used as a negative control as it does not bind to any of the immunoglobulin-binding proteins [21,24,25].

Table 2. SpLAG-anti-IgY Direct ELISA

Immunoglobulins	XOD results	Binding affinity	Coefficient of variation (CV) intra-assay (%)	CV inter-assay (%)
Duck	0.513	++	2.84	3.71
Ostrich	0.550	++	3.89	5.75
Parakeet	0.364	+	4.74	8.63
Eagle egret	0.392	+	4.01	7.17
Bantam hen	0.580	++	3.65	6.83
Pheasant	0.329	+	4.13	6.67
Chicken	0.670	++	3.03	4.88
Domestic pigeon	0.387	+	3.59	6.05
Wild pigeon	0.305	+	4.52	8.68
Goose	0.315	+	4.38	7.50
Guinea hen	0.381	+	3.12	4.85
Quail	0.396	+	4.59	8.07
Rat	0.754	++	2.95	4.50
Dog	0.995	+++	3.66	7.05
Horse	0.856	+++	5.24	9.36
Mule	0.680	++	4.96	8.88
Donkey	0.814	+++	4.62	6.01
Cat	0.655	++	3.26	5.08
Rabbit	1.058	+++	3.56	4.97
Goat	0.886	+++	4.64	7.82
Bovine	0.850	+++	4.06	6.16
Mouse	1.122	+++	3.50	5.93
Raccoon	0.907	+++	3.44	5.23
Coyote	0.875	+++	4.17	6.86
Skunk	1.259	+++	3.61	5.42
Hamster	0.751	++	5.13	9.78
Guinea pig	1.004	+++	3.57	5.36
Human	1.356	+++	4.26	6.37
Pig	1.202	+++	3.98	7.18
Dolphin	0.991	+++	4.21	6.63
Turtle IgY	0.090	-	2.88	4.34

Affinity ranges:

XOD < 0.27= - (no binding)

0.27-0.53= + (weak)

0.54-0.81= ++ (moderate)

XOD > 0.81= +++ (strong)

Table 3 shows that laying hens (74.5%) and humans (22.5%) have the highest seroprevalence of anti-salmonella antibodies among all the species. The ELISA that uses the SpLAG-anti-IgY-HRP conjugate was effective in determination of both avian and mammalian immunoglobulins. Table 4 shows the different 95% confidence intervals, which sample means fall in between. From 23.13-32.87 for laying hens, between 22.46-33.54 for humans, between 20.85-35.15 for quails, between 17.99 and 38.01 for domestic pigeons and between 15.6 and 40.4 for geese.

Table 3. Presence of anti-Salmonella antibodies in egg yolk and human serum by SpLAG-anti-IgY universal ELISA.

Species	**Positive (%)**
Human	22.5% (9/40)
Geese	10.0% (1/10)
Quail	16.0% (4/25)
Domestic Pigeon	14.3% (2/14)
Laying hen	74.5% (38/51)

Table 4. Table showing the differences in proportions between human and avian samples with 95% confidence intervals.

Statistical variables	**Laying hen**	**Human**	**Quail**	**Domestic pigeon**	**Geese**	**Difference**
Proportion of antibodies	0.745	0.225	0.160	0.143	0.100	0.117
Sample size	51	40	25	14	10	
95% Confidence interval	23.13;32.87	22.46;33.54	20.85;35.15	17.99;38.01	15.6;40.4	25.1;30.9

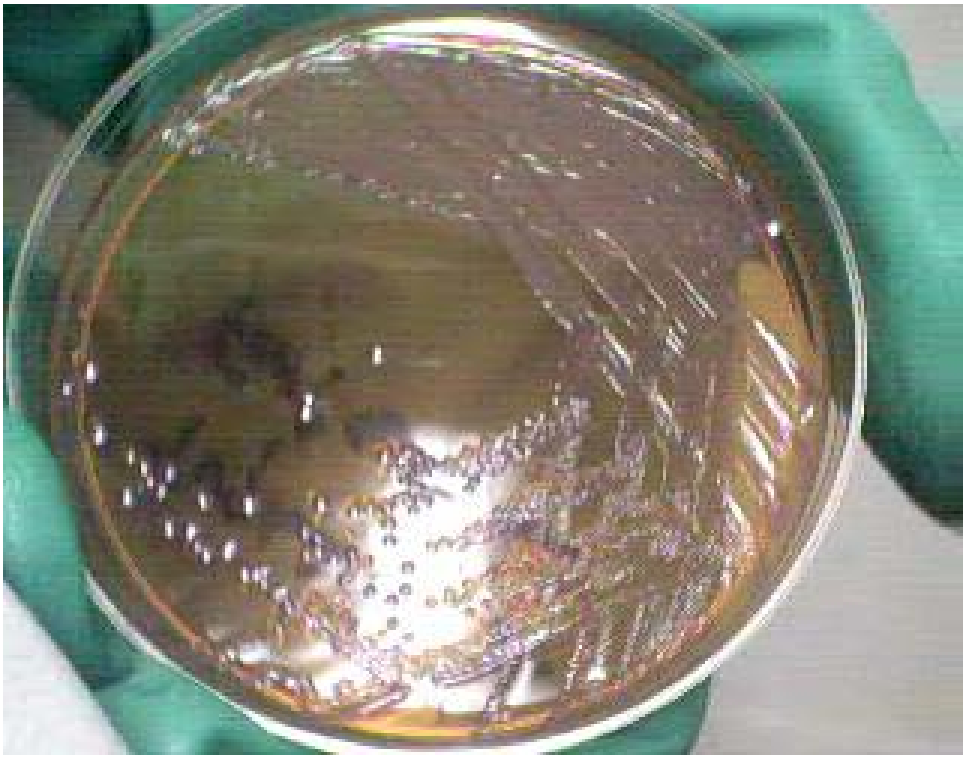


Figure 2. Typical isolated *Salmonella* colonies on differential culture media, MacConkey agar. Salmonellae are non-lactose fermenters and have a transparent appearance on MacConkey agar.

In a previous immune-epidemiological study of Salmonellosis affecting humans and chickens was reported that 11.3% of humans (6/53) and 95.3% of chicken (102/107) depicted anti-*Salmonella* antibodies in human sera and chicken egg yolks [20]. *Salmonella* is ubiquitous and chickens may be asymptomatic which has generated great health concern. This bacterium is associated with life threatening diseases among human and animals. Gastroenteritis is a disease, which may resolve itself within five days in healthy individuals. Immunocompromised individuals and the very young are at great risk as this may progress to secondary systemic complications [26].

Acknowledgements: To the Campus Research and Publication Fund of the University of West Indies, Mona Campus, Jamaica. West Indies. I have gratitude for Professors Norma Anderson and Monica Smikle from The University of the West Indies for guidance and support.

Conflict of interest: The authors declare no conflicts of interest exist.

Conclusion: Universal enzyme-linked immunosorbent assays were effective and reproducible in detecting immunoglobulins from both avian and mammalian species but the ELISA that uses SpLAG-anti-IgY-HRP conjugate only reacted with the whole panel of animal antibodies. This conjugate was further used to standardize a universal ELISA for determination of anti-Salmonella antibodies, where humans and avian species were serologically assessed.

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