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Original Research Article

Pevalence of *Samonella Species* Isolated From Powdered Infant Formula Sold in Nnewi Market

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Abstract

The continual development of Novel molecular based technologies for rapid high detection of food-borne pathogenic bacteria has made the future of conventional microbiological isolation methods termingly tenous. Report by *Salmonella species* in children is also increasing. A study on prevalence of Salmonella in PIF was conducted. A total of 10 Powdered Infant Formula consisting of 9 foreign and 1 local brand were analysed using a total sample size of 80 units.100 milk rating was done by methylene-blue reductase test, coliform analysis by serial dilution method, salmonella isolation by Bacteriological Analytical Manual(BAM) method and membrane filtration methods, while antimicrobial susceptibility by disc diffusion method. Reports obtained revealed that 80% (8) of the milk sampled belong to class 1, while 20% (2) belong to class 2. Total coliform count of different dilutions of the 10 brands sampled contained acceptable limits of coliform contamination per gram of milk. SMA infant contained lesser coliform at the higher dilutions 10^{-3}, 10^{-2} than others, 3 bacteria isolated from the 10 brands of milk include, *Salmonella species* 54 (67.5%); *(S.typhi, S.Paratyphi A,S. Paratyphi C,S. entericaand S.typhimorium), E. coli* 25 (31.25%) and *Proteus* 1 (1.25). 5 different salmonella species were isolated at different frequency using BAM and membrane filtration methods. *S. paratyphi C* 11(36.7%) and *S. paratyphi A* 14 (58.3%) were isolated using BAM and membrane filtration methods. BAM method isolated a total frequency of 30(100%). *Salmonella,* while membrane filtration 24(100%) *Salmonella species*. BAM method showed the highest recovery for *Salmonella specie* than membrane filtration method. Comparison of recovery rate between the two methods showed a statistical difference between them ($P<0.05$). Antibiotic susceptibility test show that all *Salmonella specie* was sensitive to Ciprofloxacin at (10mg) 24 (44.46%) while penicillin (30mg) had the highest resistance 54 (100%) rate. A statistical significant difference was observed between resistant and sensitive antibodies ($P<0.05$). Some powdered infant Formula sold in Nnewi are contaminated with different *Salmonella species* especially *S. Paratyphi C* and though are of class1 grade, contained acceptable coliform units/g og milk sample. Recovery rate of pathogen is best when performed with BAM method.

Keywords: Pevalence, *Samonella,* Bacteriological Analytical Manual (BAM).

INTRODUCTION

Microorganisms such as bacteria may gain entry into milk and can multiply and bring about spoilage, making processed milk unsuitable for human consumption due to rancidity, musty colors or toxin production [1]. The presence of these microorganisms usually indicates poor storage conditions or unhygienic production [2].

Today, Salmonella is a major cause of diarrheal diseases worldwide and food and water contamination have been associated as a probable transmission sources [3]. Although Samonella cannot grow outside the digestive tracts in large quantities, it can still survive in a wide range of environments if the temperature and humidity are favourable, such as in tropical countries [3].

A variety of infant formulas are available worldwide. It is available for preterm infants, full term infants and toddlers as well as specialized formulas for those infants and children with selected inborn errors of metabolism [4].

Powdered infant formular (PIF) is not a sterile product and may be intrinsically contaminated with pathogens, such as *salmonella enterica* that can cause serious illness in infants (children less than 1 year).

The potential for intrinsic contamination during production of powered infant formula(PIF) has been reviewed [5] and several survey of PIF provide an overview of the pathogen that may contaminate this infant food stuff [6].The microorganisms of greatest concern are *Salmonella enetrica* and *Enterobacter Sakazakii* [5]. A review showed recent outbreaks of Salmonella infection among infants that were attributable to contaminated PIF resulting in diarrhea and in some infants, bacteremia and meningitis.Such outbreaks occurred even when the consumed PIF appeared to be in compliance with current international standards [7].

The study was done to isolate *Salmonella species* from popularly used powdered infant milk sold in the market using two methods, identify different *Salmonella species* isolated from powdered infant formula, compare the recovery rate of the two methods (conventional method and membrane filtration method) and educate producers on the importance of stringent measures in the sterilization method and practices in infants milk production

MATERIALS AND METHOD

Study design

This study was a case study to isolate and identify salmonella species from commonly used infant milks solid in retail markets in Nnewi town.

Study area

This work was carried out in Nnewi town; Supermarkets, markets and shops within the town which serves as sources for the powdered infant formula.

Sample size

A total of 80 sample units collected from ten different brands of powdered infant formula of known sources were used for the study (that is 100g from each PIF). A total of 40 sample units were used for membrane filtration method and another 40 sample units were used for conventional method.

Calculation of sample size

Sample size was calculated using the formula:

$$N = \frac{Z^2PQ}{D^2}$$

Where N = minimum size required

P = expected prevalence rate or proportion (%)

For this study, a prevalence of 5% from similar study was used (Oni *et al.*, 2003)

Q = 1-p =1-0.05 = 0.95

(Proportion of milk free from contamination)

D = degree of accuracy

Z = the standard normal deviation for a normal distribution and was taken as 95% confidence interval which corresponds to 1.96

Ethical consideration

Ethical approval was obtained from the ethical committee of Nnamdi Azikiwe University Teaching Hospital, Nnewi.

Methods of sample collection

Sample collection entails, a total of 10 different brands of powdered infant formula comprising of 1 locally powdered infant formula (PIF) and a foreign PIF commonly used for weaning babies were purchased for the study.

These PIF include; Isomil (Netherland), Frisogold (Holland), Nan 1 (Netherland), Lactogen 1 (France), cowbell (France), My boy (Holland), SMA gold (Ireland), SMA infant (Ireland), SMA progress (Ireland), peak 1,2,3 (Nigeria).

These were bought from registered retailers, only PIF with FDA and NAFDAC registration which had evidence of expiry date were used. Twenty-five grams (25g) of four different sample units were asceptically collected from each container according the method described by [8].

These were collected at random from different spots in the milk (below, middle and on top of the milk). A total of 100g was sampled from each PIF to give a true representation of the whole milk. The collected sample units were placed in a sterile container. Aseptic techniques were employed during collection as described in [9].

Analysis of sample

Preliminary screening was done using Methylene blue reductase test () and Coliform analysis().The two standards FAO and WHO recommended methods were used for sample analysis, at the end of which the methods were compared to know the best with the highest recovery rate. Post screening analysis (microbiological analysis). Conventional method of Bacteriological Analytical manual [10]. Membrane filtration method [8].

ANALYSIS DONE

(A) Preliminary screening

- Methylene blue reductase test [11]
- Coliform analysis [11]

(B) Post screening analysis (microbiological analysis)

- Bacteriological analytical manual [12]
- Membrane filtration method [8]

Procedure for methylene blue reductase test principle of test

Aim

- To screen rapidly the quality of milk for the load of coliform and silatis.
- To screen strong reducers of methylene blue
- To check indicators of contamination

Method

- 25g of each milk sample (Isomil, Nan, lactogen 1, Frisogold, Cowbell, My boy, SMA gold, SMA infant, SMA progress, peak 1,2,3) were aseptically weighed and dissolved in 100ml of sterile distilled water.
- 10ml of each milk sample was pipetted out with sterile 10ml pipette and transferred to one screw capped sterile tube (sterile container).
- 1 ml of (1/25,000 dilution) methylene blue solution was added. 1/25,000 dilution methylene blue was prepared as seen in appendix 2
- The caps were tightened and tubes inverted second time.
- The tubes were placed in a test tube rack, the rack was placed in a water bath at 37^0c .
- These were incubated for 5 mins at 37^0c.
- The tubes were removed from water bath and inverted several times to mix again.
- The tubes were observed at 30minutes interval for 8 hrs.

Reduction

The time at which colour change in milk from blue to white was recorded. When at least 4/5 of the tube had been reached was recorded. Classification of the milk sample was done following recommendation of BAM [10] as follows:

- Class 4 milk: reduction within 30 minutes (very poor quality milk)
- Class 3 milk: reduction between 30 minutes to 2hrs (poor quality milk)
- Class 2 milk: reduction between 2hrs – 6hrs (fair quality milk)
- Class 1 milk: reduction between 6hrs-8hrs (good quality milk)

COLIFORM ANALYSIS

Principle of the test

This can be determined by testing for the presence of indicator microorganisms which are usually bacteria that, if present in large quantity may indicate that the product is not acceptable or fit for human consumption, usually an upper limit is set for the number of indicator microorganism if the counts are higher, the food is judged potentially unsafe.

Method

Coliform screening analysis of the milk was conducted to the method described below:

25g of each of the listed milk samples was aseptically weighed and dissolved in 100ml of sterile distilled water. 10ml of each milk solution (Isomil, Frisogold, Nan 1, lactogen 1, cowbell, my boy, SMA gold, SMA infant, SMA progress, peak 1,2,3) was aseptically added in a test tube and shaked 25 times. Serial dilutions of each of the milk sample were made as indicated below;

Prior to this, violet-red Bile agar (constituted VRBA) was recommended and used following instructions described in preparation method in appendix 3

- The sterile Petri dishes were labeled with respect dilutions and names of milk and date using pencil wax.
- 1ml of each milk sample was aseptically added into a sterile petri dish (10^0).
- 1 ml of each milk sample was pipetted out and added to ml saline blank and from this, 1ml was added into a Petri dish (10^{-1})
- I ml of each milk sample was pipetted out and added into 99ml saline blank from this, 1ml was added into a Petri dish (10^{-2})
- 20mls of VRBA was added to each plate and allowed to gel.
- It was incubated at 370c for 6/24hrs
- Plates that have between 25-250 colonies and were located below the surface lens shaped, deep red and surrounded by pink holes were selected.
- These were recorded as coliform counts per ml of milk
- Total coliform counts were done using the formula below:
  Number of bacteria/gram =

$$\text{Number of bacteria/gram} = \text{number of colonies} \quad \frac{x1}{\text{Diluting factor}} \quad \frac{x \quad 1}{\text{weight of sample}}$$

And were expressed as coliform unit/milk

Membrane filtration stages

Pre-enrichment (non-selective enrichment)

Principle

The test sample was initially inoculated into a non- inhibitory liquid medium to favor the repair and growth of stressed or sub lethally-injured Salmonella

arising from exposure to heat, freezing, desiccation, preservatives, high osmotic pressure or wide temperature fluctuations.

Method

25g analytical unit of each milk sample was added into 90ml of buffered peptone water respectively. (i.e. 25g of milk each in 90ml of buffered peptone water) making up to 100g from each container. These were incubated at 37^0c overnight.

Selective enrichment

Principle

Replicate portions of each pre-enrichment culture were inoculated into two enrichment media to favor the proliferation of Salmonella through a selective repression or inhibition of the growth of competing microorganisms.

Method

1.0ml of the pre-enrichment culture was aseptically pipetted into each of tetrathionate brilliant green (TBG) and selenite cystine broth and incubated at 43 ± 0.50^0c for 24 ± 2hrs at 35^0c respectively.

Selective plating

Principle

Enrichment cultures were streaked onto selective differential agar for the isolation of salmonella.

Method

A loopful of each selective enrichment culture was streaked onto selective agar using CHROMagar salmonella (CHROMagar france) to obtain well isolated cultures. Colonies of suspected salmonella species appeared as mauve colonies, blue colonies for E.coli, colourless for proteus mirabilis and these were recorded.

Purification

Principle

Presumptive Salmonella isolates were purified on macConkey agar plates.

Method

Suspected colonies were streaked onto MacConkey agar for purification. The plates were incubated at 35 ± 0.5^0c for 24±2hrs

Membrane filtration method

In this technique, a 100ml milk sample is filtered through membrane filter. The membrane with the coliform organism on it was then cultured on a pad of sterile selective broth containing lactose and an indicator. After incubation, the number of coliform colonies was counted [13]

Membrane filtration method

- An appropriate volume of sample was filtered.
- Sterile filtration apparatus was placed in position and connected to a source of vacuum with the stopcock turned off.
- The funnel was removed and the edge of the membrane filter was held with sterile smooth-tipped forceps, a sterile membrane filter was placed onto the porous disc of the filter base.
- The required volume of sample was poured into the funnel. The stopcock was opened and a vacuum not exceeding 65kpa (500mm of mercury) and the milk was filtered slowly through the membrane filter. The stopcock was closed as soon as the sample was filtered.
- The funnel was removed and the membrane filter was carefully transferred to a container containing, typically 90ml of buffered peptone water.

Sensitivity testing

Nutrient agar plates were used. Commercially prepared antimicrobial disc (optum diagnostic Nigeria) were used and placed in the nutrient agar plates after streaking the media with suspected salmonella isolates. These were incubated at 37^0c for 24hrs after which zones of clearance or inhibition were noted and recorded accordingly.

CONVENTIONAL METEHOD

Pre-enrichment (non-selective enrichment)

Using brilliant green water 25g analytical unit of each milk sample were weighed into a sterile 250ml beaker with sterile glass, 25g analytical unit were gently and slowly poured over the surface of proportionately 225ml of brilliant green water contained in sterile Erlenmeyer flask (500ml) for dilution i.e. 1:10. The loosely capped containers were incubated without mixing at 350c for 24± 2hrs. The incubated samples were tightened and shaken.

Selective enrichment

Using selective cystine broth, tetrathionate broth and Rapappaport vasiliadis soy peptone agar tetrathionate broth was prepared with the manufacturer's instructions; selenite cystine broth was prepared as described in appendix 10. Rapport vassiliadis soy peptone was prepared as described in appendix 4. Serial dilution (1 in 10) was carried out as follows;

- 1ml of the mixture was transferred to 10ml of selenite cystine (sc) broth, another 1ml of the mixture to 10ml of tetrathionate broth and another 1ml to 10ml of rapport vasiliadix and vortexed.
- These were incubated at 350c for 24±2hrs

Selective plating

Using chromagar Salmonella (chromagar france)

- 3ml loopful (10ul) of the incubated medium was streaked on chromagar salmonella
- These were incubated at 37^0c for 24hrs

- The plates were examined for the presence of suspected Salmonella.

Purification

Suspected colonies were streaked onto MacConkey agar for purification. The plates were incubated at 35±0.5^{0}c for 24±2hrs

Sensitivity testing

Nutrient agar plates were used. Commercially prepared antimicrobial disc (optum diagnostic Nigeria) were used and placed in the nutrient agar plates after streaking the media with suspected salmonella isolates. These were incubated at 37^{0}c for 24hrs after which zones of clearance or inhibition were noted and recorded accordingly.

Biochemical test for the two methods

LIA

This was used for the differentiation of Enterobacteriacea and other gram negative rods.

Procedure

A small amount of growth was harvested with a sterile (1ul) needle; the surface of the agar slant was lightly inoculated. A single stab was made into the butt of the tubes. Tubes were incubated under aerobic conditions at 36 ±1^{0}c with caps loosened. Tubes were examined and results recorded at 24hrs, 48hrs for 5-7 days (unless H_2S production occurred sooner) [14, 15].

Kligers iron agar (kia) / tripple sugar iron (tsi)

This was used for the differentiation of enterobacteriaceac and other gram negative rods.

Prodecure

A small amount of growth was harvested with a sterile (1ul) needle; the surface of the agar slant was lightly inoculated. A single stab was made into the butt of the tube. Tubes were incubated under aerobic conditions at 36 ±1^{0}c with caps loosened. Tubes were examined and results recorded at 24hrs, 48hrs for 5-7 days (unless H_2S production occurred sooner) [15].

Simmons citrate utilization test

Simmons citrate agar is a synthetic medium containing inorganic ammonium salts as a nitrogen source and sodium citrate as a carbon source. It is used to distinguish members of the enterobacteriaceae and other gram-negative rods on the basis of citrate utilization.

PROCEDURE

A small amount of growth was harvested with a sterile (1ul) loop. The surface of the agar slant was lightly inoculated. Tubes were incubated under aerobic conditions at 36 ±1^{0}c with caps loosened. Tubes were examined and results recorded at 24hrs, 48hrs for 5-7 days [14, 16]

UREA AGAR

Urea agar is used to differentiate organism based on urease activity.

PROCEDURE

A small amount of growth was harvested with a sterile (1ul) needle; the surface of the agar slant was lightly inoculated. Tubes were incubated under aerobic conditions at 36 ±1^{0}c with caps loosened. Tubes were examined and results recorded at 24hrs, 48hrs for 5-7 days [17].

Motility –indol - ornithine Agar

MIO agar was utilized to demonstrate motility, ornithine decarboxylase activity and indol production.

Procedure

A small amount of growth was harvested with a sterile needle. A single stab was made into the tube of MIO agar. The stab was made straight into the agar and stopped approximately 1cm from the bottom of the tube. Tubes were incubated under aerobic conditions at 36 ±1^{0}c with caps loosened. Tubes were examined and results recorded following overnight (18 - 24hrs) incubation [17].

SEROLOGICAL IDENTIFICATION

Widal kit was used to support the tentative identification of isolates as member of salmonella specie. Widal test is a serological technique which test for the presence of salmonella antibodies. It was used in diagnosing typhoid and paratyphoid [13].

RESULTS

Table-1a: Classification of milk highlighted according to methylene blue reductase test

S/N	NAME OF MILK	CLASS OF MILK	INTERPRETATION
1	Isomil	Class 1	Good quality
2	Frisogold	Class1	Good quality
3	Nan	Class 2	Fair quality
4	Lactogen	Class 1	Good quality
5	Cowbell	Class 1	Good quality
6	My boy	Class 1	Good quality
7	SMA gold	Class 1	Good quality
8	SMA infant	Class 1	Good quality
9	SMA progress	Class 1	Good quality
10	Peak 1,2,3	Class 2	Fair quality

The table (1a) above shows the result of classification of milk based on their quality. After methylene blue reductase test. From the result above, 2 brands of milk (Nan 1 and Peak 1,2,3) had class 2 and of fair quality while the rest of the milk belong to class 1.

Table-1b: **Frequency distribution of different classes of milk according to metylene blue reductase test**

Class of milk	Frequency	Prevalence
Class 1	8	80%
Class 2	2	20%
Total	10	100%

Table 1b represents the frequency of different classes of milk according to methylene blue reductase test. Class 1 milk had a frequency of 8 (80%) while class 2 milk had the frequency of 2(20%). No sample was observed to belong to class 3 and 4.

Table-2: Total coliform count/g of different dilutions

S/N	NAMES OF DIFFERENTS MILKS	TOTAL COLIFORM COUNTE XPRESSED IN 100 (cfu/g)		TOTAL COLIFORM COUNT EXPRESSED IN 10-1 (cfu/g)		TOTAL COLIFORM COUNT XPRESSED IN 10-2 (cfu/g)	
1	Isomil	41	(1.64)	10	(4)	0	(0)
2	Frisogold	257	(10.3)	102	(40.8)	17	(68)
3	Nan 1	250	(10.20	150	(60)	25	(100)
4	Lactogen 1	47	(1.88)	32	(12.8)	5	(20)
5	Cowbell	54	(2.16)	24	(9.6)	16	(64)
6	My boy	258	(10.3)	60	(24)	24	(96)
7	SMA gold	12	(0.48)	5	(2)	0	(0)
8	SMA infant	2	(0.08)	0	(0)	0	(0)
9	SMA progress	15	(0.6)	5	(2)	0	(0)
10	Peak 1,2,3	250	(10)	160	(64)	10	(40)

Table (2) shows the total coliform count of different dilutions expressed as cfu/g Myboy and Frisogold had the highest coliform count 10^0 257 (10.3cfu/g) while SMA infant shows the least 2(0.08)cf4/g. At 10^{-1}, peak 1,2,3 had the highest 160(64cfu/g) while SMA infant the least 0(0cfu/g) . At 10^{-2} Isomil, SMA infant, SMA progress and SMA gold had no growth at 10-2 0 (0cfu/g) while NAN 1 had the highest 25(100cfu/g). isomil, SMA Gold, SMA Infant, SMA Progress, Cowbell, Lactogen 1, All had counts below 50-100 cfu/g (the accepted maximum limit) for microbiology, above which the food is considered not fit for consumption.

Key:
Cfu/g = coliform forming unit per gram

Table-3a: Microorganisms isolated from different brands of milk

S/N	ORGANISM	FREQUENCY	PREVALENCE
1.	*Salmonella species*	54	67.5%
2.	*Escherichia coli*	25	31.25%
3.	*Proteus*	1	1.25%
TOTAL	3	80	100%

Table (3) shows the frequency of different microorganism isolated from different brands of milk, three different organisms were isolated at frequencies stated; *salmonella species* 54 (67.5%), *Esherichia coli* 25 (31.25%), *proteus* 1 (1,25%).*Salmonella* were more frequently isolated than other organism.

Key:
% = percentage.

Table-4a: Different species of salmonella isolated from the various milk brands using bam method

ORGANISM ISOLATED	ISO MIL	FRISOG OLD	NAN	LACTOGE N	COWBE LL	MY BOY	SMA GOL D	SMA INFA NT	SMA PROG RESS	PEAK 123
Salmonella typhi	11 1	0	0	0	0	2	0	1	1	0
Salmonellapar atyphi c	2	1	2	0	2	1	1	0	0	1
Salmonella enteric	1	1	0	1	0	0	2	0	0	0
SSalmonella aparatyphi A	1	0	0	2	0	2	0	2	2	0
Salmonella typhysius	0	0	0	0	1	0	0	0	0	0

Table (4a) shows the different species of salmonella isolated from the different milk brands assayed. The species are: *Salmonella typhi, Salmonella paratyphi C, Salmonella enterica, Salmonella paratyphi A and Salmonella typhisuis. From the table a total of 2 salmonella paratyphi was isolated from* Isomil*: S.typhi (1), S. enterica (1),S.typhysius (0),* frisogold*: S.typhi(0), S. paratyphi C(1), S. enterica (1), S. paratyphi A (0), S.typhysius (0),*Nan *1: S.typhi (1), S. paratyphi C(2), S. enterica (0), S. paratyphi A (0), S.typhysius (0), Lactogen: S.typhi(0), S. paratyphi C(0), S. enterica (1), S. paratyphi A (2), S.typhysius (0),*cowbell*: S.typhi(0), S. paratyphi C(2), S. enterica (0), S. paratyphi A (0), S.typhysius (1),*my boy*: S.typhi(2), S. paratyphi C(1), S. enterica (0), S. paratyphi A (2), S.typhysius (0),*SMAgold*: S.typhi(0), S. paratyphi C(1), S. enterica (2), S. paratyphi A (0), S.typhysius (0),* SMA infant*: S.typhi(1), S. paratyphi C(0), S. enterica (0), S. paratyphi A (2), S.typhysius (0),*SMA progress*: S.typhi(1), S. paratyphi C(0), S. enterica (0), S. paratyphi A (2), S.typhysius (0),*Peak 1,2,3*: S.typhi(0), S. paratyphi C(1), S. enterica (0), S. paratyphi A (0), S.typhysius.* less number was isolated than others using this method.

Table-4b: Frequency of different Salmonella species isolated using BAM method

s/n	Salmonella specie	Frequency	Prevalence%
1	*S. typhi*	4	13.3
2	*S. enterica*	5	16.7
3	*Salmonella paratyphi A*	8	26.7
4	*Salmonella paratyphi C*	11	36.7
5	*S. typhisius*	2	6.6
Total	5	30	100

Table 4b shows the frequency distribution of different salmonella species isolated using BAM method. A total of 5 different salmonella species (*S. typhi, S. enteric, Salmonella paratyphi A, Salmonella paratyphi C, S.typhisius*) were isolated. *S.typhisius* has the lowest prevalence 2(6.6%) while S.paratyhi C the highest, *11(36.7).*

Key: s= salmonella
% = percentage.

Table-5a: Isolation of different species of salmonella from various milk brands using membrane filteration method

ORGANISM ISOLATED	ISOMI L	FRISOGOL D	NA N	LACTOGE N	COWB ELL	MY BO Y	SMA GOL D	SMA INFAN T	SMA PROGRE SS	PEA K 123
Sal. Typhi	0	0	1	0	0	1	0	0	0	0
Sal. paratyphi C	4	2	2	3	2	2	0	1	1	0
Sal. Enterica	0	0	0	0	0	0	0	0	0	0
Sal..paratyphi A.	1	0	0	0	1	1	0	0	0	0
Sal.typhimurium	0	0	0	0	0	1	1	0	0	0

Table (5a) shows different species of salmonella isolated from different milk brand. The species are: *Salmonella typhi, Salmonella paratyphi C, Salmonella enterica, Salmonella paratyphi and Salmonella typhimurium. From the table a total of (5) salmonella paratyphi C was isolated from* Isomil*: S.typhi (0), S. paratyphi C (4), S.enterica (0), S. paratyphi (1), S. tyhimurium (0),*frisogold*: S.typhi (0),S. paratyphi C (2), S.enterica (0), S. paratyphi (0), S. tyhimurium (0),Nan:S.typhi (1), S. paratyphi C (2), S.enterica (0), S.*

*paratyphi (0), S. tyhimurium (0),Lactogen: S.typhi (0), S. paratyphi C (3), S.enterica (0), S. paratyphi (0), S. tyhimurium (0), cowbell: S.typhi (0), S. paratyphi C (2), S.enterica (0), S. paratyphi (1), S. tyhimurium (0), my boy: S.typhi (1), S. paratyphi C (2), S.enterica (0), S. paratyphi (1), S. tyhimurium (0), SMA gold: S.typhi (0), S. paratyphi C (0), S.enterica (0), S. paratyphi (0), S. tyhimurium (1),SMA infant: S.typhi (0), S. paratyphi C (1), S.enterica (0), S. paratyphi (0), S. tyhimurium (0), SMA progress: S.typhi (0), S. paratyphi C (1), S.enterica (0), S. paratyphi (0), S. tyhimurium (0), peak 1,2,3: s.typhi (0), S. paratyphi C (0), S.enterica (0), S. paratyphi (0),S. tyhimurium (0).*No *Salmonella enterica* was isolated with this method.

Table-5b: Frequency of different salmonella specie isolated using Membrane filtration method

S/N	SALMONELLA SPECIE	FREQUENCY	PREVALENCE (%)
1	*Salmonella paratyphi A*	14	58.3
2	*Salmonella paratyphi C*	6	25
3	*Salmonella typhi*	2	8.35
4	*Salmonella typhumurium*	2	8.35
Total	4	24	100

Table 5b shows the frequency of different salmonella species isolated using membrane filtration method. A total ot four salmonella species consisting of four different species was isolated. *Salmonella paratyphi A* had a frequency of *14 (58.3%), Salmonella paratyphi C 6(25%), Salmonella typhi 2 (8.35%), Salmonella typhumurium 2 (8.35%).were isolated at frequencies stated above. S.paratyphi A* was more commonly isolated than the rest 14 (58.3%).

Key:
S= salmonella; % = percentage.

Table-6: comparison of bam and membrane filtration methods used in isolation of different species of salmonella from the various milk brands

			MEMBRANEFILTRATION									Total
			ISO MIL	FRISO GOLD	NAN	LACTOGEN	COWBELL	MY BOY	SMA GOLD	SMA INFANT	SMA PROGRESS	
BAM METHOD	1SOMIL	Count	5	0	0	0	0	0	0	0	0	5
		Expected Count	1.0	.4	.6	.6	.6	1.0	.2	.2	.2	5.0
	FRISOGOLD	Count	0	2	0	0	0	0	0	0	0	2
		Expected Count	.4	.2	.2	.2	.2	.4	.1	.1	.1	2.0
	NAN	Count	0	0	3	0	0	0	0	0	0	3
		Expected Count	.6	.2	.4	.4	.4	.6	.1	.1	.1	3.0
	LACTOGEN	Count	0	0	0	3	0	0	0	0	0	3
		Expected Count	.6	.2	.4	.4	.4	.6	.1	.1	.1	3.0
	COWBELL	Count	0	0	0	0	3	0	0	0	0	3
		Expected Count	.6	.2	.4	.4	.4	.6	.1	.1	.1	3.0
	MY BOY	Count	0	0	0	0	0	5	0	0	0	5
		Expected Count	1.0	.4	.6	.6	.6	1.0	.2	.2	.2	5.0
	SMAGOLD	Count	0	0	0	0	0	0	1	1	1	3
		Expected Count	.6	.2	.4	.4	.4	.6	.1	.1	.1	3.0
Total		Count	5	2	3	3	3	5	1	1	1	24
		Expected Count	5.0	2.0	3.0	3.0	3.0	5.0	1.0	1.0	1.0	24.0

X^2 =32.104, p = 0.000 (p >0.005, significant)

Table (6) shows the comparison of recovery rate of salmonella species from of the milk samples using 2 methods. BAM method showed the highest recovery for S. paratyphi C 11(36.7%). A statistical difference was observed when compared between the two in comparison of recovery rates of salmonella species ($p<0.05$).

Table-7a: Invitro disc diffusion susceptibility pattern of the different *salmonella* organism isolated from the Ten P.I.F.

Antibiotics Gram Negativ	Streptomycin (30ug)	septrin (30ug)	Tarivid (10ug)	Pefloxacin (10ug)	Augmentin (25ug)	Ciprofloxacin (10ug)	Gentamycin (10ug)	Ampicilin (30mcg)	Nalidic acid (30mcg)	Penicilin (30mcg)
salmonella n=(54) Sensitive(s) Resistant (R)	17 (31.48)% 37(68.52)%	12 (22.2)% 42(77.8)%	19 (35.1)% 35(64.9)%	6 (11.1)% 48(88.9)%	12 (22.2)% 42(77.8)%	24(44.4)% 30(55.6)%	10(18.5)% 44(81.5)%	2 (3.7)% 52(96.3)%	6(11.1)% 48(88.9)%	0(0)% 54(100)%

Table 7a shows the invitro disc diffusion susceptibility pattern of the different Salmonella organism isolated from PIF. Penicillin (30ug) showed the highest resistance for all Salmonella species isolated 54 (0%) out of the total of 54, Ciprofloxacin (10ug) 24(44.46), showed the highest sensitivity to other drugs at different concentration showed varying levels of sensitivity as stated. P value showed that there was a statistical significant difference between the resistant and sensitive antibiotics as most of the isolates showed reistance to most of the drugs tested X^2 = 486, DF = 17,P = 0.000(P<0.05, Significant). There was more resistance observed in drugs used.

Effects of antibiotics to sensitivity and resistance of some salmonella spp

Expected Count	5.3	18.7	24.0
Count	0	30	30
Expected Count	6.7	23.3	30.0
Count	0	10	10
Expected Count	2.2	7.8	10.0
Count	0	44	44
Expected Count	9.8	34.2	44.0
Count	0	3	3
Expected Count	.7	2.3	3.0
Count	0	51	51
Expected Count	11.3	39.7	51.0
Count	0	9	9
Expected Count	2.0	7.0	9.0
Count	0	45	45
Expected Count	10.0	35.0	45.0
Count	108	378	486
Expected Count	108.0	378.0	486.0

DISCUSSION

Many reporters have proven that powdered infant formula is not sterile and may be intrinsically contaminated with pathogens, especially Enterobactericae such as Salmonella specie and cause serious illness in infants. Although a death of knowledge exists for developing countries concerning incidences of salmonellosis in infants especially in PIF. It is the most common and widely reported cause of food borne disease [18].

Almost all (80%) except in two cases (20%) (Nan 1 and peak 1,2,3)were of good quality class I, this could be as a result of the fact that most of them were foreign milks produced outside Nigeria with better quality control measures. The two class II milks, peak 1, 2, 3 and Nan I are produced and packaged in Africa.

In the study, all milk brands had indicator coliforms isolated from them up to 10^{-2} dilutions except Isomil, SMA gold, SMA progress and SMA Infant. SMA Infant had organisms at only 10^{0} dilutions (0.08cfu/ml) so it can be presumed that the batch assayed is the best of the lot. Isomil, Lactogen 1, cowbell, SMA gold, SMA Infant, SMA progress were the brands that had counts below the acceptable limit recommended in milk bacteriology so are considered the fit milks. According to [11], the maximum acceptable total coliform count in milk is 50-100cfu/g. The values obtained in the listed milk are below this. This could be because most of the milks are foreign packaged milks with full authenticated authorized registration. This could mean that most of the recommended manufacturing practices are met with. The listed milks also belonged to class 1 in the methylene blue reductase test. Other microorganisms like *E. coli* 25 (31.25%), *Proteus* (1.25%) and *Salmonella species*54 (67.5%) were isolated from the milk sample, Mahami *et al.* [19] isolated 6 organism: *Enterobacter, Proteus, S. typhi, Ent.*faecalis, *Staphylococcus aureus, Staphylococcus epididymis* from 6 categories of branded and unbranded milks sold in Accra Ghana. Bergstrom [20] in a commonly based study carried out in India on bacterial contamination of nutrient concentrates in infant milk isolates *E. coli* 64%, *Enterococci* 26% from the infant milk studied, 67% of milk sampled had at least one organism. No *shigella* or Salmonellosis was found. This is likely because infant milk is not a sterile product. Licani, in [21] discovered that Salmonella survived during spray drying and subsequent handling of skimmed milk powder. That means that some organisms were found to resist some pasteurization processes. Some *Enterobacteria* have been found to contaminate sources or points of processing plant during pauagy FAO/WHO [6]. Moreover; FAO/WHO [6, 22] also reported that powered infant formula is readily available as a supplement or replacement for breast milk. Though it is heat-treated during processing, but unlike liquid formula product is not subjected to sufficient treatment to make the final packaged product commercial sterile.

Salmonella species was more frequently isolated than other organisms in all the milk brand 54(67.5%).In table 4b,using BAM method ,a total frequency of 30 *Salmonella* consisting of 5 *Salmonella species* at different percentage was obtained with BAM method and a total frequency of 24 Salmonella consisting of 4 different *Salmonella species* with membrane filtration method, this is in line with the findings of FAO/WHO [6, 22] which stated that salmonella have been identified as the greatest concern with PIF, this is because FAO stated that this non sterile product is not subjected to sufficient treatment to make the final package of product commercially sterile .

Though all milk samples had the coliform counts at lower acceptable level. The highest total coliform count was in Nan at 10^{-2} (100cfu) while the least was 0cfu from most milk. The total coliform count showed that all the milk samples were fit for human consumption. The highest acceptable limit of total coliform count in milk microbiology is 100cfu [11].

In the current work, a prevalence of Salmonella species was found to contaminate some of the milk formulas. Salmonella is a dangerous pathogen, it causes clinical infection when ingested at a dose of 10^5 – 10^8 (10^3) in some cases. High incidences of Salmonellosis among infants have been reported. Nestle has recalled voluntarily Nesquick Chocolate powder that may be contaminated with some UPC and production codes, and demanded that the affected product should not be consumed but returned to its point of purchase [12]. Lican [21] also proved that Salmonella survived during spray drying and subsequent handling in skimmed milk powders. *S. Thompson* and *S. typumurium* was found to be more resistant to heat storage than other Salmonella species when stored at different temperature of 25-50^{0}C for 8 weeks. Though a rapid destruction occurred in the first 12 weeks of storage, it was followed by a reduced rate of reduction at 35-25^{0}C, reduced Salmonella numbers were obtained in 4-8 weeks, such storage would not make heavily contaminated powders Salmonella negative in 8 weeks. Beckers *et al*. [23] also worked on the suitability of artificially contaminated milk powder as a substrate for Salmonella reference sample and their stability under different storage temperature, especially at 4^{0}C, they found milk powder as a suitable material for reference sample through a reconstitution steps were needed to be included in the standard Salmonella isolation procedure.

In all cases, most of these PIF must have been contaminated through post processing processes like packaging, through poor environmental management practices or addition of some pre and post processing. Salmonella as danger in milk diary product was issued as a warning by the department of agriculture in 1967 especially in manufacturing plants after the *Salmonella new-brunswick* outbreak in the US in milk powder. In 1977, it was detected in Casein from a western Victoria plant and in 1978 from 4 more plants and another *Salmonella bredeney* outbreak in Infant milk powder that same year. Dryer insulation was implicated as the source of contamination especially damp insulation [24]. Different pre-enrichment media, incubation temperatures and time, selective plating media also affect the rate of recovery of *Salmonella specie* in powdered infant formula. A collaborative study involving 19 laboratories study to validate enrichment on modified Rappaport Vassiliadis medium for rapid detection of motile Salmonella in dried milk products was done. MSRV

Method was compared with the AOAC culture method for detecting Salmonella in non-fat milk powder, whole milk powder, whey powder, casein powder and butter milk powder, MSRV sensitivity was 100 % and AOAC 99%. MSRV method was therefore adopted first action by AOAC International. Bolderdijk and Milas [25]. In the present work, isolation with MSRV enrichment broth was also used and yielded a lot of *Salmonella specie.*

Bacteriological Analytical Manual method (BAM) recovered more of *S. paratyphiC* 11 (36.7%) than other specie while membrane filtration method recovered more of *S. paratyphi A* 14 (58.3%) than other species. In both cases, serovar *S. typhi* was recovered 4(13.3%) and 2 (8.35%) respectively. Comparative statistical analysis of recovery rate of Salmonella species from milk sample using the 2 methods showed that BAM method showed the highest recovery for *Salmonella paratyphi C* (36.7%). A statistical difference was observed when both recovery methods were compared ($P<0.05$). Many attempts have been made to device the best method for recovering Salmonella from foods and dairy products. New methods are described regularly. El-shamy [11] evaluated two enrichment broths, three plating media and Elisa technique for recovery of Salmonella from raw dairy products. Brilliant Green Agar and X.L.D (Xylose Lysine Deoxycholate Agar) were compared with the raw chrome Salmonella for recovery Salmonella from 160 dairy products with enrichment in Rappaport Vassiliadis and Tethrathionate broth. TEGRA unique Salmonella, Elisa test was also used, only 1 sample was positive for Salmonella which appeared in each of chrome Salmonella and XLD agar enrichment using RV not TT and was found to have a sensitivity and specificity of (100%, 92.45%),(100%, 93.71%) and (0%,100%) for each of chrom Agar Salmonella, XLD and Brilliant green agar respectively. TECRA unique Salmonella test yielded highest sensitivity and specificity (100%, 100%) in all, In this work, RV media broth yielded more Salmonella than TT broth. The reason is because not all media are universally sensitive for detecting Salmonella from all sample types and some do not support the growth of certain serovars of Salmonella. RV is currently

recommended by Wallace for the recovery of Salmonella from low and high contaminated foods at 43^0C while TT incubated at 35^0C is indicated for food with low microbial load and at 43^0C for high microbial load.

Invitro disc susceptibility studies of the different isolates of Salmonella species obtained from the PIF samples showed that 9 drugs were susceptible for isolates at different level. No isolate was sensitive to Penicillin (PN) (30mg) 0(0%) (100%) resistance was therefore recorded for the drug. Ciprofloxacin (40mg) was susceptible to 24 (44.4%) of the isolated Salmonella specie, it was the drug most sensitive to the different species of Salmonella isolated while other drugs for which the different species of Salmonella were susceptible to include Tarivid (10mg) 19(35.1%), Streptomycin (30mg) 17(31.48%), Septrin (30mg) and Augumentin (20mg) 12 (22.2%), Gentamycin (10mg) 10(18.5%), Pefloxacin (10mg) and Nalidic acid (30mcg) 6 (11.1%) and Ampicillin (30mcg) 2(3.7%) respectively. Mahami *et al.* [19] in a prevalence study of antibiotic-resistant bacteria in milk sold in Accra reported that all isolates obtained *(Salmonella typhi, E. faecalis, S. aureus, S. epididemis, E.coli, Klebsiella, Enterobacter and Proteus* were multi resistant to Ampicillin, Chlorophenicol, Gentamycin, Ceftriaxine and Ciprofloxacin and this showed a statistical difference between the resistant and sensitive antibiotics ($P<0.00$) as more resistance was noted for the different *Salmonella species* isolated.

CONCLUSION

Most popular brands of PIF sold in Nnewi markets contain acceptable coliform count/g of microorganisms stipulated by FAO, belong to class 1 and are contaminated by *Salmonella species* 54(67.5%) more than other Enterobacteriaceae. A great number of the isolated species were resistant to microorganisms with 100% resistance showed by Penicillin, Ciprofloxacin was more sensitive than other drugs for the different *Salmonella species* isolated.

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