International Journal of Agricultural Research and Food Production
ISSN: 2536-7331 (Print): 2536-734x (Online)
Volume 5, Number 3, September 2020
http://www.casirmediapublishing.com

MOLECULAR DETECTION AND ENUMERATION OF GRAM-POSITIVE PATHOGENS IN SOME POWDERED INFANT FOODS IN IKOT EKPENE METROPOLIS, NIGERIA

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ABSTRACT: The present study was conducted to detect and enumerate Gram positive bacteria in some powdered food sold within Ikot Ekpene metropolis using the polymerase chain reaction(PCR) based method. Ten different samples were obtained from different supermarkets and pharmaceutical stores and were tested for Gram positive bacteria. The results obtained using the culture based method indicated that samples were contaminated by Gram positive bacteria (spore forming and non-spore forming). The PCR based method was further used for identification and the results revealed the identity of *Lactobacillus sp, Listeria monocytogenes, Bacillus cereus, Clostridium perfringens and Staphylococcus aureus.* The use of molecular method has facilitated pathogen detection in powdered food as they have posed some health challenges to consumers. From the result obtained, greater emphasis are placed on good manufacturing practices with careful attention to heat inactivation of bacterial spores, hygienic conditions, sanitation of equipment and processing plants. Implementation of food safety policy by government with the objective of reducing food-borne illness among consumers is recommended. This will ensure microbial quality, food safety and standards of powdered infant foods. Further study should be carried out on bacterial spores, their ability to survive during processing in food industries and the step to kill the vegetative cells.
Keywords: Gram positive bacteria, contamination, Polymerase chain reaction, pathogen.

INTRODUCTION

Infant powdered food is a food product consumed during early life, infancy and when the mother begins to introduce foods other than breast milk. These foods product generally represent a rich source of nutrients and contain ingredients from various origins, hence carrying a potential risk of exposure to foodborne pathogens. Infants are more liable to be infected by foodborne illnesses; this is

because their immune systems are not fully developed (Townsend and Forsythe, 2008).

The advantages of powdered milk over liquid milk are better keeping quality, less storage space, and low shipping costs (Robert *et al.*, 2015). Powdered food is made by the removal of water content from liquid milk. Removal of water is necessary to reduce water activity for the prevention of microbial growth. Powdered milk has a maximum shelf life of about 3 years while skim milk has a maximum shelf life of about 6 months (Flegam and Oluwaniyi, 2015). Food manufacturing industries are contemplating on adding value to powdered infant milk with emphasis on microbiological pathogens, quality, sensory characteristics , physical and chemical properties which are mainly concerned with the moisture content , fat, total protein, and non-protein nitrogen, lactose, titratable acidity, ash, and other nutrients such as calcium (Laszlo, 2007). The thermophilic organisms have ability to produce extremely heat resistant spores and can have significant economic consequences when they exceed specification limits. This may result in microbial contamination of infant powdered foods (Anup and Rupesh, 2012).Microbial pathogens which are of major concern in dried milk include *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella*. These organisms may remain viable in milk powder for long period of time, and resume growth when the powder is reconstituted and stored at favourable temperature (Hafsa *et al.*, 2013).

The bacterial contaminant in powdered foods includes species of the class Bacilli many of which are capable of forming endospores (Checinska *et al.*, 2015). Other Bacteria have also been found to contaminate powdered dairy products visa vis *Clostridium halophilum, Klebsiella oxytoca* (Buehner *et al.*, 2015), *C. perfringens, C. septicum, C. novyi/haemolyticum, C. sporogenes* (Barash *et al.*, 2010), *Staphylococcus aureus* (Zhang *et al.*, 2015), and *Cronobacter sakazakii* (Minami *et al.*, 2012). *Bacillus cereus* which is a spore

former can survive various drying and heat-treatments such as pasteurisation in the food industry (Andersson *et al.*, 1995). *Clostridium perfringens* is found in a variety of places including; food, water and air. The highly resistant to temperature, vegetative cells, and dormant spores makes it survive under extreme condition (Novak and Juneja, 2002).*C. perfringens* is present in a natural environment, a contaminant of food, causing food poisoning (Miah *et al.*, 2011; Tseng and Labbe, 2000; Kunene *et al.*, 1999; Miah *et al.*, 2011). Of the spore-formers identified in powders, specific representatives of *Clostridium* spp. and *Bacillus* spp. are the most worrying from a food safety point of view.

Enterobacter sakazakii , is an opportunistic pathogen associated with severe foodborne illnesses in infants and neonates, such as fatal neonatal meningitis, sepsis, death and necrotizing enterocolitis(Bar-Oz *et al.*,2001;Van Acker *et al.*, 2001).Listeria monocytogenes has the ability to survive in different temperatures in powdered foods and this is a problem within food industries. *Listeria monocytogenes* is a facultative pathogenic saprophyte. It can cause a severe disease, listeriosis, which is currently considered to be one of the leading food-borne diseases worldwide. *Listeria monocytogenes* has been recognized as an important transmission route leading to human listeriosis, becoming a public health problem. However, research has found that Listeria is able to survive the drying process and has been found in powdered food products (Lammerding and Doyle, 1990). This is particularly interesting as Listeria is a non-spore forming organism. And its ability to survive as a vegetative cell poses quite a serious problem within the food industry. *Staphylococcus aureus* has been noted to be able to survive for a long time in powdered food products. However in products such as dried infant formula milk, the contamination normally occurs from non-sterile spoons or from human contact with the milk. However, research conducted showed that *Staphylococcus* can contaminate

powdered infant food from the day of opening the product and can multiply (Umoh *et al.*, 1985).

Staphylococcus aureus is a pathogen associated with serious community and hospital-acquired diseases. It has low nutritional requirements and widely exists in nature. Staphylococcus is the leading cause of food borne pathogens causing food poisoning. This foodborne pathogen is considered as one of the world's leading causes of disease outbreaks related to food consumption, being responsible for a variety of manifestations and diseases (Jamali *et al.*, 2015).Lactobacillus is an important genus of lactic acid bacteria. More *Lactobacillus sp.*is classified as probiotics causing health effect in the body (Sade and Lassila, 2016). *Lactobacillus* is a Gram positive non spore forming rod shaped bacteria. Lactobacilli have been extensively studied for their molecular biology in order to improve their specific beneficial characteristics (Pouwel and Leer, 1993). PCR based method of identification of microorganism has some advantages as it is rapid and reliable method of producing large number of DNA for identification. So therefore, the purpose of this research was to detect, characterize and enumerate Gram positive pathogens present in powdered infants' foods using the molecular based method.

MATERIALS AND METHODS

Study Area

The study was conducted in Ikot Ekpene Local Government Area, Akwa Ibom State in the Southern part of Nigeria. The city has an area of about 166km2, with GPS coordinates of Latitude: 5.18o N, and Longitude: 7.71 E.

Source of Sample Collection

A total of Ten (10) samples of powdered infant foods were obtained randomly from different Pharmaceutical stores, supermarkets/ retail outlets in Ikot Ekpene. Sterile techniques were applied and

samples were transported immediately to microbiology laboratory for microbiological analysis.

Preparation of Samples

All samples were serially diluted; 9 ml of distilled water were added to 1 g of each sample and thoroughly mixed and was homogenized by shaking for 1 minute. Tenfold serial dilution was carried out in all the samples. After dilution 0.1 ml of the appropriate dilutions were used to inoculate Nutrient Agar, Plate count Agar, Blood agar, Baid parker agar, Tryptone Sulphite Agar. The media were prepared according to the manufacturer's instruction. Pour plate method was employed and the culture plates for isolation were incubated at 37 °C for 24 hours for enumeration of colonies.

Characterization and Identification of Bacteria Isolates

Bacterial isolates were characterized and identified based on their morphological and cultural characteristics. Confirmatory and identification was based on biochemical test carried as described by (Chessbrough, 2006). The following biochemical test were carried out such as gram's staining, spore test, catalase, coagulase, oxidase, indole, citrate test, urease test and sugar fermentation test (glucose, galactose, sucrose and maltose).

Genetic Characterization of Isolates

DNA Extraction of Isolates

Fresh colonies of the bacteria isolated from different brands of powdered foods were grown on BHI broth. A sterile loop was used to resuspend one colony in 1 ml of TE buffer and was supplemented with lysozyme (10 µl/ml). The cells were then incubated at 37 °C for 15 min. The solution was then boiled at 95 °C for 15 minutes, it was then allowed to cool, and was centrifuged at 13,000 g for 5 min. 10 µl sample was used as DNA template in each PCR .

Column purification of DNA

After 24 hrs of culturing, a colony of the solution was transferred to a centrifuge tip with 15 µl sterile water. It was incubated at 90 °C for 7 min. The solution was transferred to Zymo spin IC column for fast spinning and was centrifuge for purification at 13,000 g for 1 min. 100 µl of wash buffer was added to the column and was then centrifuged for three times at 5000 rpm for 0.5 min. The column was then pulse spin at 13,000 g for 10 sec. 9 ml of sterile water was pippeted into the column. 1.5 ml microcentrifuge tube with the column placed on it was centrifuged at 13000 g for 1 min to remove the DNA.

GES extraction of DNA

An overnight culture of each organism was obtained in BHI broth.1.5 ml of the culture was placed in 1.5 ml micro centrifuge tube and pulse spin at 13,000 g for 1 min. The supernatant was eluate, the pellet was obtained and suspended in 1 ml lysis buffer. 50 mg/ml lysosome was then added to the lysis. Samples were incubated for 30 min at 35 °C .0.5 M guanidine thiocyanate, 0.1 N EDTA, 0.5 % N- lauroyl – sarcosine sodium salt was added , mix, and incubated at 27 °C for 5 min and allowed to cool. The lysis was then placed on the ice for 2 min and 0.25 ml of 7.5 M ammonium acetate was added, vortexed and incubated on ice for 10 min. Following incubation, the solution was centrifuged at 13,000 g for 10 min. 850 ml of the topmost part were removed and placed in a clean Eppendorf tube. 0.5 ml of isopropanol was added to the solution, mix was centrifuged at 13,000 for 5 min and the supernatant removed. The pellet was washed three times in 70 % ethanol and dried at 37 °C for 10 min. After drying, the pellet was resuspended in 50 µl TE buffer.

Polymerase chain reaction (PCR) amplification of plasmid cpa DNA gene of Clostridium

Genomic DNA was extracted using DNA purification kit and PCR for amplification of *cpa* gene.. 25 µl reaction volume of 10 x buffer, 20

nM Cl_2, 10 nM $MgCl_2$ and 0.2 % (w/v gelatine, 500 mM KCl, and 10 nM dNTPs. Two set of primers F- GCTAATGTTACTGCCGTTGA and R- CCTCTGATACATCGTGTAAG for amplification of 100 bp of the targeted *cpa* gene. 25 ul mixture of graded water was added and 0.5 ul Taq DNA polymerase was added . After initial denaturing at 93 °C for 5 mins,25 cycles at 94 ° C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min were performed and a final extension step at 72 °C for 8 min. PCR products were visualized using ethidium bromide stains at agarose gel electrophoresis

Polymerase chain reaction (PCR) amplification of 16S rDNA gene of Bacillus

Fresh colonies grown on MYP Agar were randomly picked. A PCR method combined with amplified rDNA restriction was used to identify *Bacillus cereus*. 10 µl of the DNA extracted was used in a 25 µl PCR reaction volume. 0.8 mM of dNTPs, 1 x buffer solution, 1.5 mM $Mgcl_2$, Two set of primers F-, (TCACCAAGGCRACGATGCG), and R- (CGTATTCACCGCGGCATG) were used for amplification of 100 bp of the targeted gene and 1µl of Taq DNA polymerase. It was then centrifuged and placed in a thermocycler machine to amplify the segments of the DNA. The PCR was carried out at an initial denaturing step of 94 °C for 2 min, followed by30 cycles at 94 °C for 30 sec, 52 °C for 30 sec and 72 °C for 2 min and the final extension step at 72 °C for 5 min. PCR products were visualized using ethidium bromide stains at agarose gel electrophoresis.

Polymerase chain reaction (PCR) amplification of 16S rDNA gene of Staphylococcus aureus

Characterization of *Staphylococcus aureus* was carried out using PCR method. 20 µl reaction volume consisting 10 µl extracted DNA from cell culture was inoculated into BP Agar. 1 µl of 60 Mm Kcl , 3 µl of Tris- Hcl, 2 µl of 0.1 % Triton X – 100,2 µl of 2.5 mM $Mgcl_2$ and 1 µl of each *Staphylococcus aureus* primer; F-,(AATCTTTGTCGGTACACGATATTCTTCACG),and R -

,(CGTAATGAGATTTCAGTAGATAATACAACA) was added with 0.1 µl of dNTPs together with Taq DNA Polymerase of 0.5 µl. Deionized water of 15 ml was added and centrifuged and placed in a thermocycler machine for PCR. After the initial denaturing at 94 °C for 5 min, it was followed by 25 cycles PCR amplification at 94 °C for 1 min, and 55 °C for 1 min and the final extension of 72 °C for 1 min. PCR products were visualized using ethidium bromide stains at agarose gel electrophoresis.

Polymerase chain reaction (PCR) amplification of 16S rDNA gene of *Listeria monocytogenes*

Fresh colonies of bacteria grown on Brain-Heart Agar (BHA) was randomly picked. Sterile loop was used to resuspend one colony in 1 ml of TE buffer. 20 µl PCR reaction containing 0.1 µl of 10 x buffer, 0.1 µl of 0.2 Mm mixture of dNTPs, 1.5 µl of 1.5 mM $Mgcl_2$ was prepared in microcentrifuge tube 0.5µl of primers F-, (GCTAATACCGAATGATAAGA), and R- (AAGCAGTTACTCTTATCCT) and 1.5 µl of LIS –R was added to the tube and 5 µl of deionized water. 10 µl DNA extract and 0.1 µl of TaqDNA polymerse was then added to PCR mixture. The reaction mixture was amplified in a thermocycler with the following PCR conditions. The temperature for annealing was 94 °C for 5 min, followed by 5 cycles of 95 °C for 30 sec, 53 °C for 45 s, and 72 °C for 45 s. This was followed by30 cycles of 95 °C for 45 s, 58 °C for 30 s and 72 °C for 1 min. The final extension step was at 72 °C for 5 min. The PCR products were separated using ethidium bromide stains at agarose gel electrophoresis.

Polymerase chain reaction (PCR) amplification of 16S rDNA gene of *Lactobacillus*

The PCR amplification of Lactobacillus sp was carried out according to the method carried out by Wu *et al.*, 2006. 16S rRNA gene was performed using primers F – TCTAAGGAAGCGAAGGAT and R- CTCTTCTCGGTCGCTCTA 273 bp. 25 µl multiplex PCR reaction was

prepared containing 0.1 µl of 10 x PCR buffer. 0.1 µl of 0.2 mM dNTPs and 1.5 µl of 1.5 mM $Mgcl_2$ and distilled water. The parameters for the PCR were followed. Initial denaturing at 94 °C for 5 min, 35 cycles of 95 °C for 45 s, 53 °C for 45 s, extension step of 72 °C for 1 min and final extension at 72 °C for 4 min. PCR products were visualized using ethidium bromide stains at agarose gel electrophoresis.

RESULTS AND DISCUSSION

Consumption of powdered foods has been a health problem because of the health effects of consuming this contaminated food products. Fig 1 shows PCR identification for *Clostridium*. The results show a clear band of target gene of 324 bp yielding positive results of cpa toxins in the sample. *Clostridium* produces enterotoxin which causes food poisoning if present in powdered infant foods, and is consumed by infants can cause food poisoning and can result in food borne illness (Scallen *et al.*, 2011).

Fig 2 shows the PCR identification for *Listeria monocytogenes*. A multiplex PCR reaction involving the use of primers that target the DNA regions in the Listeria genus was found in *Listeria monocytogenes* to produce a band of 287 bp. Thus showing that samples were contaminated with *L. monocytogenes* (Amagliania *et al.*, 2004). The results indicated that the primers have a high affinity for the correct target sequence and are specific for L. monocytogenes. Fig 3 shows the PCR identification for *Bacillus*. This method has recently been used for the detection of enterotoxins related to *Bacillus cereus* in powdered infant foods (*Wu et al., 2006).* The culture based method indicated that the 10 samples showed pink colonies in the MYP agar and were considered been contaminated due to their characteristic features of cell morphology, motility, spore forming rods on the media. The 16S rRNA genes were not shown and the PCR identification of *Bacillus* was incomplete, and no identification was carried out. Nevertheless, since 90 % of the

organisms formed characteristic colonies on the growth medium, their identity was certain (*Guven et al., 2006*). *Bacillus* has spores that are found in food products, so therefore it is important that investigation of these pathogens in powdered food be examined. PCR based method have been developed to enumerate food borne pathogens' in powdered foods as they are the major cause of food poisoning when this products are consumed. Fig 4 shows the PCR amplification screening of 16S rRNA gene of *Staphylococcus sp*. Lane 2 is the positive control. After testing the product with PCR, a band at 108 bp was seen, a clear indication of sample contamination. Identification of *Staphylococcus aureus* was also carried out using the culture based methods. The organisms had the characteristics cell and colony morphology as well as catalase and coagulase tests .Fig 5 shows the PCR amplification 16S 23S rRNA gene of *Lactobacillus sp*. This investigation indicates that some samples were contaminated. The PCR method was able to solve the problem of poor identification that is usually associated with the identification of Gram positive bacteria.

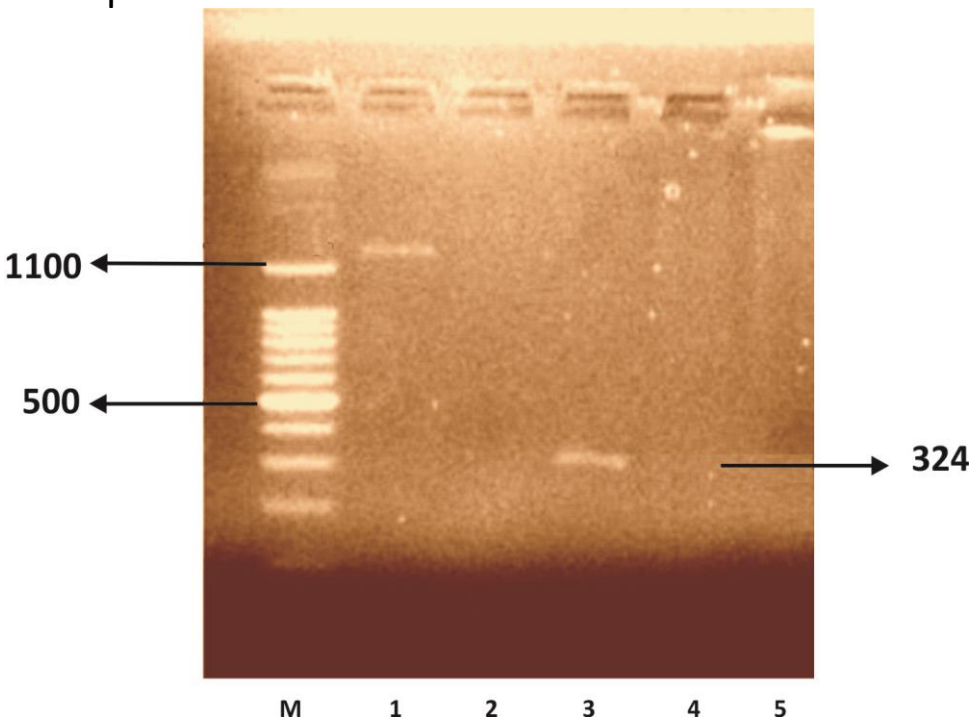

Fig. 1. Agarose gel electrophoresis result of Clostridium perfringens with PCR amplification products specific for *Clostridum perfringens*

cpa toxin. Lane M = molecular maker(promega 100bp ladder, lanes 1 – 4 contains PCR products, lane 5 is the negative control.

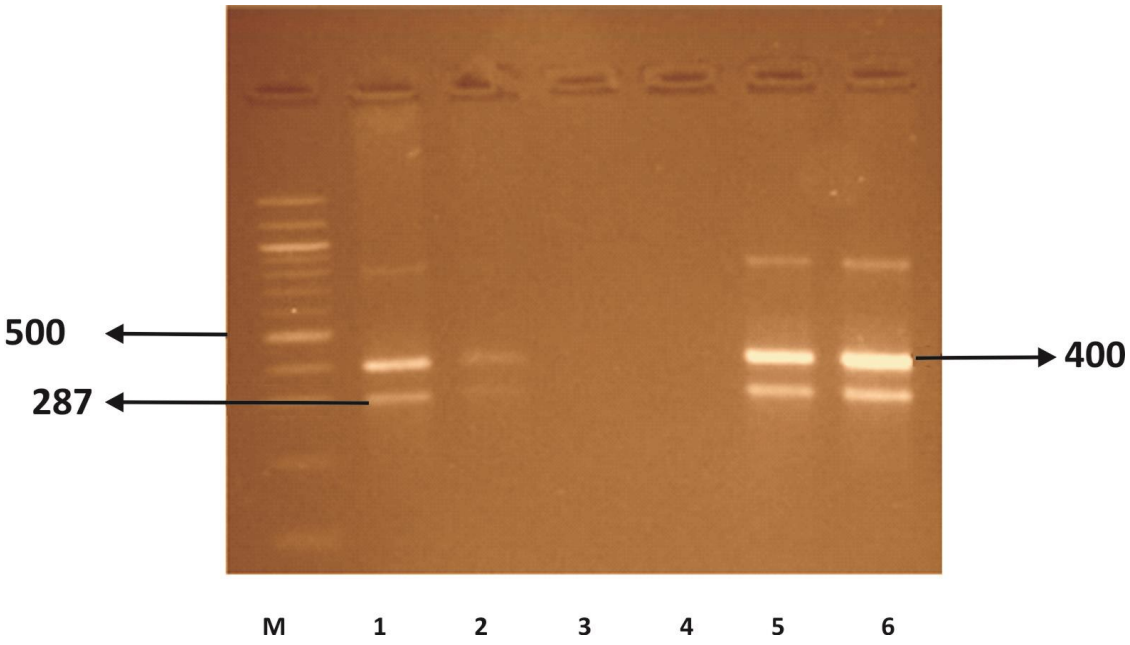

Fig 2 . Agarose gel electrophoresis result of *Listeria monocytogenes* with PCR amplification products. Lane M = molecular marker promega 100 bp ladder. Lane 1 – 2 and 5-6 are laboratory strains of *Listeria monocytogenes*(ATCC 23074 and NCTC 7973). Lane 3-4 are the negative control.

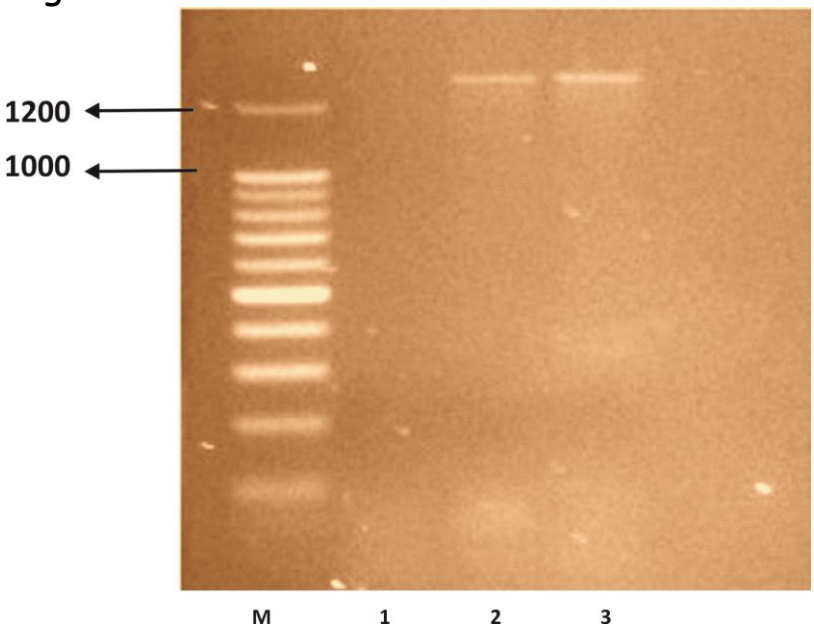

Fig. 3 .Agarose gel electrophoresis result of *Bacillus cereus* PCR amplification products. Lanes M , marker promega 100bp ladder, Lane 1 is the negative control, lane 2 and 3 contains the PCR products gained from the laboratory strains of *Bacillus cereus*(ATCC 211 B)

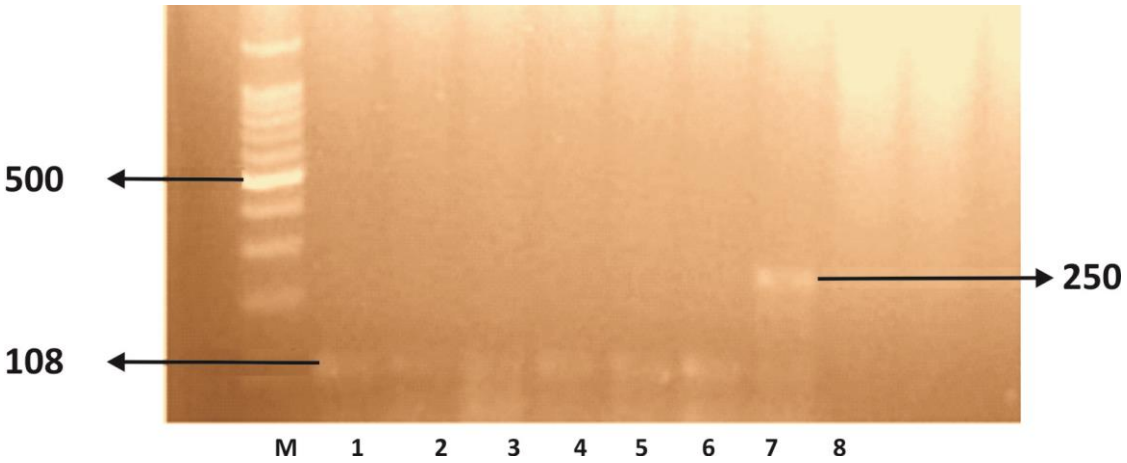

Fig. 4. Agarose gel electrophoresis result of *Staphylococcus aereus* with PCR amplification products specific for *S. aureus*. M= molecular marker, (Promega 100bp ladder). Lane 1 is positive control (*S. aureus*, strain-12100 DNA) lane 2-7 consist of PCR products amplified from isolates from products and lane 8 is negative control.

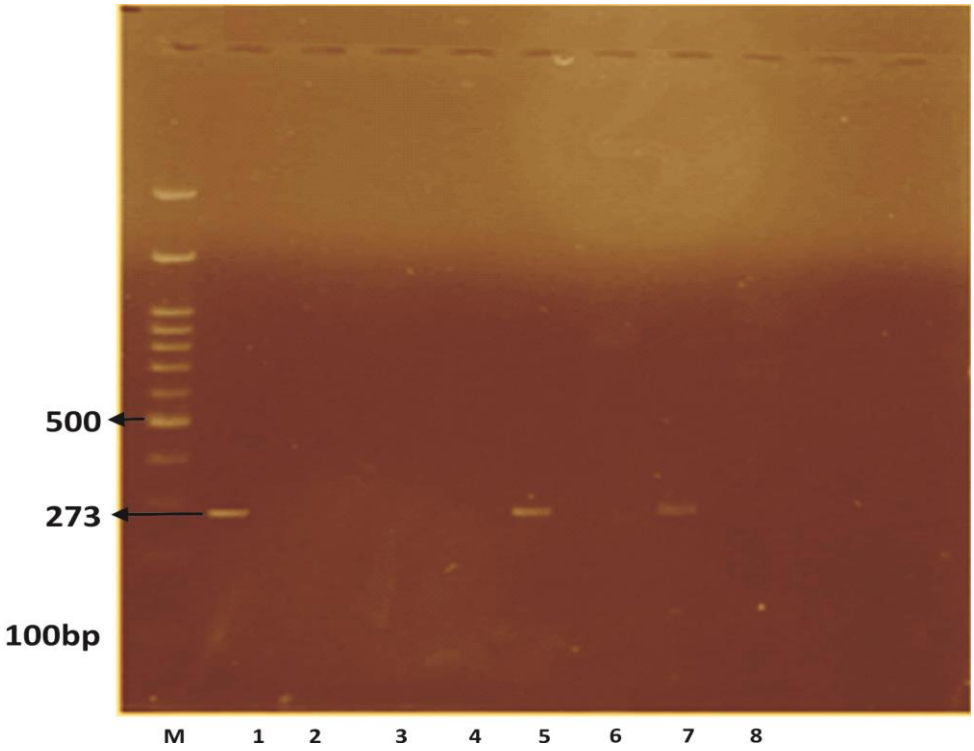

FIG. 5. Agarose gel electrophoresis result of *Lactobacillus* with PCR amplification products specific for *Lactobacillus sp.* M= molecular marker, (Promega 100bp ladder). Lane 1 is positive control, lane 2,4, 6 consist of PCR products amplified from isolates products and lane7 and 8 is negative control .

CONCLUSION

The molecular technique used in this study is suitable for the identification and enumeration of Gram positive bacteria in powdered infant foods. In the present study, it has revealed that samples obtained were contaminated by pathogens, hence unfit for human consumption. Therefore proper manufacturing process be carried out to ensuring free bacterial contamination of infant powdered foods. The ten products were contaminated by different Gram positive pathogens indicating a poor manufacturing process. PCR method is a vital technique for the detection of potential pathogens in powdered foods and is successfully applied in laboratories.

Acknowledgments: ***This work would not have been possible without the financial support of the Tertiary Education***

Trust Fund(Tetfund) Abuja , Nigeria who have been supportive and have provided the necessary financial support to complete this research work.

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