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Contribution of *InvA* Gene PCR to Recovery of *Salmonella* spp. Strains

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Authors' contributions

This work was carried out in collaboration among all authors. Authors BS, KFH, YKR, CKJ, KS and DM designed the study and wrote the protocol. Author BS wrote the first draft of the manuscript and managed the analyses of the study. Author GGB revised the first draft of the manuscript. Authors KKAA and YKR managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: *Salmonella* infection remains a major public health concern worldwide, contributing the economic burden of both industrialized and developing countries through the costs associated with surveillance, prevention, and treatment of disease. This zoonosis has a harmful health and economic impact in terms of death, hospitalization, and destruction of livestock on farms. To adapts the means of control and prevention against this threat, the phenotypical characterization of *Salmonella* strains, both those recently identified and those which have been conserved for a long time, is necessary. So, the aim of this study was to check quality of the *salmonella* strains first stored in storage tubes in NRC of *salmonella* of Institute Pasteur of Côte d'Ivoire.
Place and Duration of Study: This study was done in Institute Pasteur of Côte d'Ivoire between July 2019 and October 2019.

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Methodology: A total of 56 tubes used to store *salmonella* strains with few or no agar were analyzed to assess presence of Salmonella. The strains were first cultured in broth and then on selective agar (Hecktoen) medium and nutrient agar. Then, the Salmonella-specific InvA gene was directly detected in the tubes.
Results: The results obtained showed that the quality of the *Salmonella* strains initially conserved for at least 10 years had clearly deteriorated because none of them had been cultured after enrichment and culture on agar media. However, 73.21% of tubes not containing storage agar have traces of *Salmonella* DNA followed by molecular identification.
The preponderant contaminating bacterial flora was represented by Gram positive.
Conclusion: These results should encourage all laboratories to proceed immediately with the quality control of their strain collections for excellent biobanking.

Keywords: Salmonella; conservation; quality control; PCR.

1. INTRODUCTION

Salmonella infection remains a major public health concern worldwide, contributing to the economic burden of both industrialized and developing countries through the cost associated with surveillance, prevention, and treatment of disease [1,2,3,4].

In most developing countries, human salmonellosis causes between 20 and 25 % of deaths per year [5]. In addition, the emergence of multi-drug resistant of *Salmonella* strains reduces all efforts to combat this threat worldwide.

The rapid spread capacity of *Salmonella* strains has been documented [6], demonstrating the need for a reorganization of existing control and prevention measures and the urgent development and implementation of new ones.

Faced this worrying situation, our country has taken measures, including the establishment of a national reference center for *Salmonella* by inter-ministerial order No. 393 of 21 June 2006 designating the list of national references centers of the Pasteur Institute of Côte d'Ivoire (IPCI) in infectious diseases.

The main mission of national reference center is to ensure epidemiological surveillance of *Salmonella* infections.

This surveillance is carried out to detect epidemics and their outbreaks early and to assess trends in the spatial and temporal evolution of these bacteria.

This mission is made possible by the establishment of a collection of strains. Thus, the national reference center (NRC) of *salmonella* at Pasteur institute has proceeded to conserve the strains of *salmonella* of diverse origins isolated and identified in application of this interministerial order throughout the national area.

These *Salmonella* strains were stored in storage agar placed at laboratory temperature without the addition of paraffin or glycerol to the surface of the storage agar.

This type of conservation, despite its many disadvantages, has the advantage of being inexpensive and does not require heavy and sophisticated equipment for its implementation. It is therefore used by many laboratories with limited resources.

The creation in 2017 of a Center for Biological Resources (CeReB) at Pasteur Institute, a decade after the creation of the establishment of the national reference centers, leads to transfer of strains from centers to the new conservation methods for better storage over very long periods of time.

This transfer of strains requires the satisfaction of a certain quality criteria.

However, the examination of the tubes used to keep these strains showed many of them contained few or no storage agar.

The problem arising from this situation is to determine if the strains of *Salmonella* stored in the tubes used to keep there and conservation tubes are kept there and in which condition are they?

This study was carried out with the aim of checking quality of the *Salmonella* strains first stored in storage tubes.

2. MATERIALS AND METHODS

2.1 Location of Study and Sample Preparation

This work was conducted at the National Reference Center for *Salmonella* and molecular Biology Platform of the Pasteur Institute of Côte d'Ivoire. The tubes used to store *salmonella* strains with few or no agar were included in this study. Then, fifty-six (56) tubes were selected for this study from a random sample of seventy-six (76) tubes (Fig. 1).

2.2 Laboratory Methods

Two laboratory techniques were used in this study including culture and molecular methods.

2.2.1 Culture of strains

To verify the presence of *Salmonella* strains that were initially conserved in the storage agar, strains were cultured in broth (Enrichment phase) and on solid media before a morphological identification.

2.2.2 Enrichment phase

Briefly, 1.5 mL of brain and heart broth (OXOID, France) was added to each storage tube not containing storage agar to allow the growth of the stored strains. As from this first H0 batch, several H3, H6, H9 and H24 tubes containing 1.5 mL of brain and heart broth were inoculated according the "cascade" method and then placed respectively into 37°C in incubator for 3 hours, 6 hours, 9 hours and 18-24 hours in aerobic conditions.

2.2.3 Isolation and identification of isolates

Two types of bacterial growth media were used for isolating *Salmonella* strains from the enrichment broths contained in the storage tubes: a selective medium and a nutrient medium. Thus, each tubes H3, H6, H9 and H24 were streaked onto Hecktoen selective medium (OXOID, France) and nutrient medium and incubated at 37 °C for 24 hours in aerobic conditions. Presumptive colonies presented on Hektoen selective medium and those present on nutrient medium were purified by subculture on nutrient agar (OXOID, France) and identified by Gram's staining reaction. Pure colonies were identified using biochemical tests including motility, indole, urease, mannitol, lysine decarboxylase, citrate, Kliger Iron Agar to determine H_2S and gas production, lactose and glucose fermentation, oxidase, and catalase.

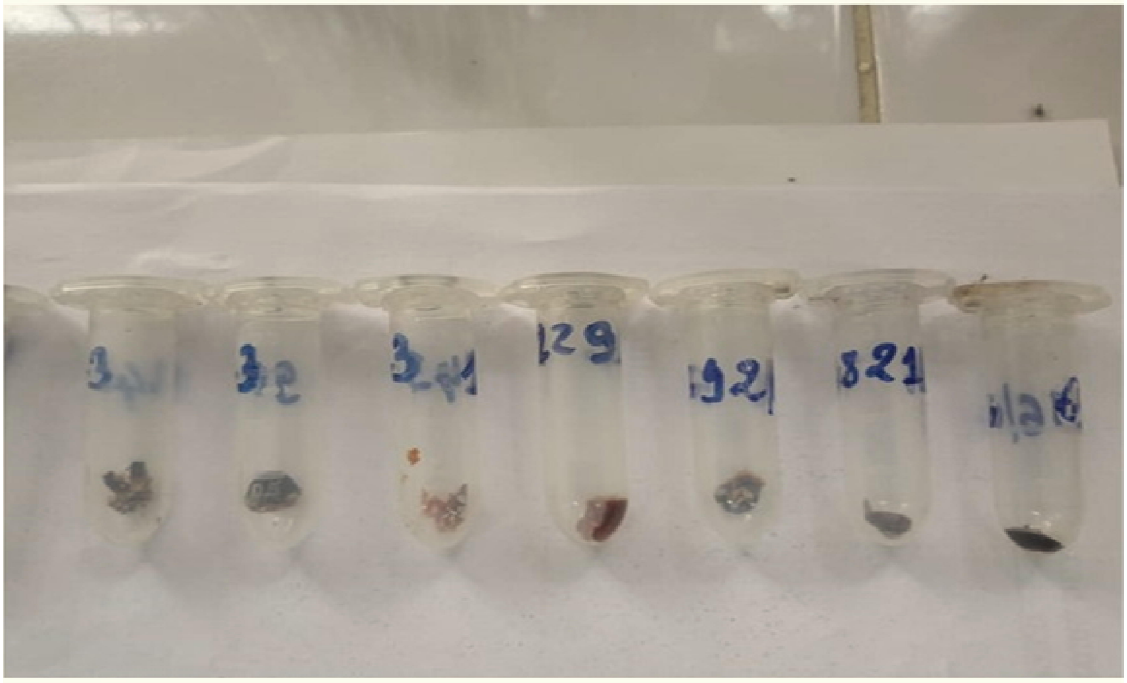

Fig. 1. Storage tubes containing little or no conservation agar

2.2.4 DNA extraction and PCR

DNA extraction from each enrichment broth was performed according to method described by SamBrook and Russel [7] with slight modifications. Briefly 1 ml of the enrichment broth stored at 4°C was centrifuged at 14000 rpm for 10 min at 4°C. Then, the pellet was suspended in 500 µL of pure water (Nuclease Free Water, Promega, Madison USA) before incubation at -20 °C for 15 minutes and then at 95°C for 15 minutes at 500 rpm followed DNA extraction with phenol + chloroform + isoamyl (25:24:1) and precipitation with ethanol. Success or failure of the DNA extraction was evaluated by electrophoretic migration and the DNA was stored for later use at -20°C.Molecular identification was performed as previously described [8] using PCR amplification of the *invA* gene encoding an invasion protein in Salmonella. Sequences of primers used for gene amplification are INVA-1 5' ACAGTGCTCGTTTACGACCTGAAT 3' and INVA-2 5' AGACGACTGGTACTGATCGATAAT 3'. PCR was performed in final volume of 50 µL MIX containing a 0.5 µL µL of each dNTP (10 mM) (Promega, Madison, USA), 3 µL of MgCl2 (25 mM), 5 µL of buffer 5X DNA taq polymerase (Promega, Madison, USA) 0.2 µL of Taq polymerase and 0.75 µL of each primer *INVA* (10 µM) (Promega, Madison, USA).PCR consisted of incubating for 5 minutes at94°C followed by 35 cycles of 94°C for 30 seconds, 56 °c for 30 seconds and 72°c for 2 minute.DNA extracted from reference strains provided by the national food institute collection (DTU FOOD) were used as positive controls for PCR and a reaction mixture without DNA extract served as a negative control. All PCR reactions were performed in thermal cycler (Gene Amp PCR system type 9700, Thermo scientific, Dardilly, France).The PCR products were stained with a 0.3% solution of SIBR Safe GREEN (Invitrogen, USA) and were visualized under UV light on gel DOC system imager (Biorad, France) after gel electrophoresis on 1.5 % agarose at 120 volts / cm for 30-45 minutes.

3. RESULTS AND DISCUSSION

3.1 Conditions of Storage Tubes

Seventeen (30.36%) storage tubes not containing storage agar had a shelf life of 16 years, 32 (57.14%) of these tubes had a shelf life of 15 years and only one (1.79%) had a recent shelf life (Table 1). However, 10.71% of these tubes had no known shelf life. Among, the tested strains, 45 (80.35 %) belonged to the serovar Hadar, one (1) strain was Senfterberg, one (1) was Derby and one (1) isolate was Typhimurium. However, the serovars of eight (8) strains were not indicated. On the over hand, most of tested strains were of animal origin (Fig. 2). These statistics show a clear inequality according to the origin of the strains received and serotyped by the NRC of *Salmonella* of Côte d'Ivoire. The low proportion of isolates of human origin in the collection could reflect an inefficiency of the NRC in the surveillance of this pathogenic bacterium in the population. This low proportion could also be due to a lack of Knowledge of the NRC of *Salmonella* and its importance in the surveillance of this bacterium in our country even if though *Salmonella* is a major cause of diarrheal disease in Côte d'Ivoire. According to Coulibaly et al. [9], the low proportion of strains of human origin could also be due to the reduced number of partner clinical bacteriology laboratories, the difficulty of access to these laboratories and the limited financial resources for the complete serotyping of the strains received between years 2006 to 2010. The high proportion of non-typhi *Salmonella* such as Hadar, of avian origin, confirms the statistics obtained.

3.2 Identification of Bacterial Strains

3.2.1 Morphological and biochemical identification of isolates

On the selective medium (Hecktoen agar), only two enriched tubes showed colonies with presumptive phenotypic characteristics of *Salmonella* spp (Fig. 2). Identification from the biochemical characteristics of these colonies showed Gram-negative rodsand some biochemical characteristics including production of hydrogen sulfide (H_2S), urease and lactose degradation that did not satisfy the characteristics of *Salmonella* genus. The results showed that no strains present in the tubes used to preserve strains containing little or no conservation agar were cultured with Salmonella-specific biochemical characteristics. The reason for this can probably be explained by the long conservation period of these strains.

Indeed, many studies have shown that after 6 months of storage by the agar storage method at room temperature in the laboratory, the number of viable microorganisms drops drastically to zero [10]. Yet, the minimum storage life of the *Salmonella* strains in this study is between 12 and 14 years.

In addition, although no conserved strain was cultured with Salmonella-specific biochemical characteristics, this study showed that two conservation tubes yielded bacterial colonies on the selective medium. Analysis of these bacterial colonies revealed that they degraded lactose and did not produce hydrogen sulphide, two unusual phenotypic characteristics in Salmonella.

This observation is according to this reported by Robson et al. [11], who state that loss of biochemical traits is not uncommon in microorganisms stored for several years [11]). Moreover, morphological identification of bacterial colonies on nutrient agar indicated that the conserved strains were contaminated mostly by Gram positive cocci (30 %) and by other Gram negative rod-shapedbacteria (12 %).

3.2.2 Molecular identification

Among, the 56 samples analysed, 41 (73.21%) were positive for invA gene justified by presence of PCR product with a size of 244 bp as this observed for the positive control represented by a reference strain of *Salmonella thyphimurium* DT104 from the National Food Institute (Lyngby, Denmark) (Fig. 2).

This high positivity shows the specificity and broad spectrum of the Inva gene in the identification or search for *Salmonella* in a biological product as shown in the study by Yao [12].

In view of this molecular result, the two *Salmonella* strains presumed on the basis of phenotypic characters were analyzed in order to better elucidate the previous hypothesis. It was found that one of these strains did not contain the *Salmonella* InvA gene, so it was not a Salmonella. On the other hand, the other strain contained the InvA gene, and was therefore a Salmonella. Thus, this strain, which in order to survive this long shelf-life, had to change some of its phenotypic traits, such as lactose degradation and non-production of hydrogen sulphide.

Table 1. Distribution of empty storage tubes according to storage years

Storage periods	Number of tubes (N)	Percentage of empty tubes (%)
2003	17	30,36
2004	32	57,14
2007	1	1,79
Unknown	6	10,71
Total	56	100

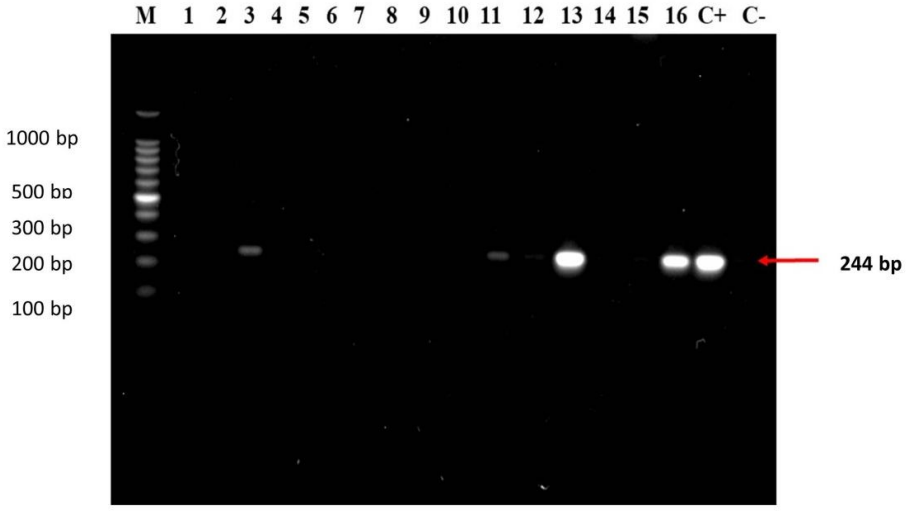

Fig. 2. Electrophoretic profile obtained after amplification of the InvA gene in stored *Salmonella* strains

M : molecular weight marker; 1-16 : extracted DNA samples; C+ : salmonella typhimurium strain DT104.; C- : negative control

Table 2. Distribution of preservation tubes according to bacterial culture positivity

Culture	Hecktoen (% of strains)	Nutrient agar (% of strains)
After 3 hours	0 (0)	0 (0)
After 6 hours	0 (0)	2 (3,56)
After 9 hours	0 (0)	26 (46,42)
After 24 hours	2 (3,56)	46 (82,14)

As for the other strain, it could probably be a contaminating bacterium. In fact, our study showed that only 7.14% of the empty storage tubes containing a single type of bacterial colony (Gram-negative rod-shaped), compared to 92.86% of the tubes containing at least 2 types of colonies, or 1 type of colony that were either Gram-Positive cocci or Gram-Positive bacilli or rodsor a combination of the 2 and with sometimes Gram-negativerods.

The presence of non-*Salmonella* in the preservation tubes could be a probable contamination of the strains. Wasas et al. [13] have also found an 80% contamination rate in the preservation tubes after only 6 months of storage.

Based on the analysis of morphological and biochemical characteristics, combined with the results of molecular identification of the bacterial strains storage indicated that *Salmonella* strains still contained in the storage tubes for at least 12 years were either viable but not cultivable or simply dead except one, which was viable and cultivable but whose phenotypic characteristics had changed. However, 73% of the storage tubes included in this study contained *Salmonella* DNA.

4. CONCLUSION

In conclusion, among, the 56 tubes containing *salmonella* strains initially conserved in agar, the presence of *salmonella* DNA was detected in 73.21% of cases. While only two conservation tubes waswere cultured on selective medium and one strain has confirmed as *salmonella* spp. by PCR, the biochemical characteristics werehave altered. Our study also showed a high contamination rate (92.86%) of the preservation tubes. These results should encourage all laboratories to proceed immediately with the quality control of their strain collections for excellent biobanking.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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