**Comparative study between the sequencing of 16sRNA gene and –traditional identification methods of some bacterial Pathogens associated with a systemic bacterial infection**

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**Abstract**

Poultry health problems of chickens are usually accompanied by bacterial systemic infections causing severe losses in flocks, sick birds were systemically could be infected by several bacteria regardless of the clinical signs. 16s RNA sequence is a modern approach for the diagnosis of bacterial infection, especially of multiple causative agents. Ten field cases suffering from systemic infections have been examined for bacterial causes, using conventional isolation and 16S rRNA sequencing, The ten sequenced samples were compatible with the results of isolation; six samples were similar to *E.coli* species with identity% = 97-99%, while it had similarity% < 96% with *Salmonella enterica*. While the other samples showed variable identity ranging from 94-99% with *Salmonella enterica, E. coli,* Enterobacter spp and Klebsiella spp

 In conclusion, although conventional isolation is the golden standard method for bacterial diagnosis, however, the 16s RNA sequencing has elaborated excellence in diagnosis and identification of bacterial infection, concerning the time consuming, detection of multi-caused infections and sensitivity.

Keywords: 16sRNA, sequencing, poultry.

1. **Introduction**

Systemic bacterial infections are caused by several acute bacterial pathogens such as Salmonella hens systemically infected with Salmonella Enteritidis may lay eggs contaminated with *Salmonella Enteritidis* ( Gast 2013), *E coli* which produce more severe signs in systemic infections than localized forms and may also spread through yolk sac ( *Nolan, et al.,* 2013)*, Pasteurella* ( Glisson, et al., 2013) and *Klebsiella* ( Abdul-Aziz & Barnes 2013)

Rapid diagnosis of bacterial pathogens can facilitate early accurate identification of causative agents of field problems and help in the prevention of diseases dissemination and in screening of bacteria in surveys and outbreaks (Kysela, et al .,2005; Clarridge III 2004; Watts et al. 2017), in addition, it may play a role in the detection of unique pathogenic microorganisms ( Nakamura et al. 2008). furthermore, it is important in the case of slow-growing or fastidious bacteria which can’t be easily subcultured

( Srinivasan et al. 2015; Watts et al. 2017; Salipante et al. 2013 ), also Culturing bacteria consumes a longer time in comparison to advanced molecular techniques, all fore-mentioned reasons lead to empirical treatment (Lehmann et al. 2008; Srinivasan et al. 2015)**.** Development of 16 sRNA sequencing which can avoid all previous obstacles in traditional culture techniques as it is a fast technique, takes a shorter time ( Salipante et al. 2013; Srinivasan et al. 2015 ); despite the high expense, it may replace the ordinary culturing technique in some laboratories ( Nakamura et al. 2008 )

Recently, 16 sRNA (small subunit) found in all prokaryotes, it is part of 30 s ribosome subunit, also, it has constant and variable regions which are considered a genetic marker for phylogenetic identification between different microorganisms ( Clarridge III 2004). One of the greatest directives of microbiology is Bergey’s Manual of Systematic Bacteriology, which uses 16sRNA as a tool for bacterial identification ( Werner et al. 2012 ). 16 sRNA is used as a tool for screening gut microbiota( Liu et al. 2019; Ocejo, et al., 2019 ), it is well known that gut microbiota plays a critical role in digestion, metabolism, and health of poultry( Yeoman et al. 2012; Ocejo, et al.,2019 ), despite, there isn’t a distinct identification of healthy cecal microbiota, which can be changed or altered by different factors as age, ration composition, housing environment, some pathogens as *Salmonella, E coli* & *Campylobacter* especially *Campylobacter* which is mainly transmitted to human through poultry( Ocejo, et al., 2019 ).

Empirical treatment of bacterial infection leads to the development of antimicrobial resistance, so we are in bad need to minimize the time for bacterial identification till applying sensitivity test and choosing the suitable antibiotic for the causative agent

( Kerremans et al.,2008).

This study aims to compare the accuracy of the 16S rRNA gene as a molecular technique for bacterial identification to traditional bacterial isolation methods from poultry flocks suffering from systemic infections, and for detection of culture-negative samples, showing the advantages & disadvantages of 16S rRNA as an alternative technique for microbiological identification

1. **Materials and Methods**
	1. **Collection of samples**

Ten cases suffering from systemic infections were collected from broiler flocks that have been submitted to RLQP for bacteriological examination. During postmortem examination, intestine, and internal organs (heart, liver) were collected aseptically for isolation and 16s RNA identification of the causative agents.

* 1. **Bacterial isolation by conventional cultivation**

Samples were examined bacteriologically to identify the different bacterial species that may cause the cases of systemic infections and diarrhea such as *E. coli*, *Salmonella*, *Pasteurella* spp, & Clostridiosis

Isolated and identified *E. coli* according to ( *Nolan, et al.,* 2013 **)**.

Isolation and identification of *Salmonella* spp according to ( ISO 6579-1).

Isolation and Identification of *Pasteurella* spp. according to (Glisson, et al., 2013 )

Isolation of *Clostridium perfringens*: according to (Tessari et al.2014).

Isolation of Klebsiella: according to ( Hamza et al., 2016)

* 1. **Molecular identification by 16sRNA sequence:**
		1. **Extraction of bacterial DNA:**

DNA extraction from samples was performed using the QIAamp DNA Mini kit (Qiagen, Germany, GmbH) with modifications from the manufacturer’s recommendations. Briefly, 200 µl of the sample suspension was incubated with 20 µl of proteinase K and 200 µl of lysis buffer at 56OC for 10 min. After incubation, 200 µl of 100% ethanol was added to the lysate. The sample was then washed and centrifuged following the manufacturer’s recommendations. Nucleic acid was eluted with 100 µl of elution buffer provided in the kit.

* + 1. **Amplification of 16sRNA gene by polymerase chain reaction:**

The propagation of the 16sRNA gene in this study was applied by Thermo two step PCR kit (Thermo scientific), with primers designed by (Lagacé et al., 2004), the sequence of primers are 16S rRNA-F: AGAGTTTGATCMTGGCTCAG and 16S rRNA- R: TACGGYTACCTTGTTACGACTT. The polymerase chain reactions were done according to the manual instruction of the PCR kit as following: 12.5ul PCR master mix, 1 ul of each primer with concentration 20pmol, 5ul of DNA then complete the total volume to 20 ul with PCR grade water. The amplification condition ran as initial denaturation at 95c for 5 min for one cycle, 40 cycles for 3 following steps: denaturation at 95c for 45 sec., primer annealing at 56c for 45 sec. and polymerization at 72c for 1 min., completed with 1 cycle of final extension step at 72c for 10 min.

The products of PCR were separated by electrophoresis on 1.5% agarose gel (Applichem, Germany, GmbH) in 1x TBE buffer at room temperature. The expected molecular weight for the electrophorized product is 1100 bp.

* + 1. **Gene sequence and analysis for amplified 16s RNA gene:**

The amplified PCR products with the appropriate size were subsequently purified by using a QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The purified PCR products were directed for sequencing reactions using a Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) according to the manufacturer, the reaction product was purified by exclusion chromatography in DyeEX 2.0 Spin Kit. The recovered materials were sequenced using a 3500 XL DNA Analyzer (Applied Biosystems).

Analysis of the 16s RNA gene sequences of the target bacteria in this study was accomplished by blast on gene bank (NCBI), then the alignment of the sequences was done by Bioedit software.

1. **Results:**
	1. **Ethical approval**

Handling of chicken during sample collection was in accordance with the regulations for the care and welfare of examined animals and approved by the Animal Care Committee of the Animal Health Research Institute

* 1. **Conventional isolation by culturing:**

As shown in Table 1, seven out of ten examined samples (70%) were positive for E.coli, followed by four samples (40%) have possessed Salmonella spp, and only one sample was positive for Klebsiella

* 1. **Nucleotide sequencing of 16sRNA gene:**

We depend on the BLAST tool in Genebank (NCBI) to get the results for the nucleotide sequence identity of the tested samples. Genebank (NCBI) is a huge world database that provides enormous sequence data for different organisms including the 16s rRNA sequences of different bacteria. The ten sequenced samples were compatible with the results of isolation. In detail, we have got six out of ten samples similar to *E.coli* species with identity% = 97-99%, while these sequences showed similarity% < 96% with *Salmonella enterica*. Also, we have got two out of ten sequences similar to the *Salmonella enterica* with identity =94-97%, however, the same samples were also similar to the *E.coli* species with percent= 96%. The K4 was *Klebsiella* in isolation, but in sequence blast result it was similar to Enterobacter spp. (99%), *E. coli* (97%), *Salmonella* spp (96%-99%), and Klebsiella spp (97-98%). the K7 sample have got mixed infection with E.coli and Salmonella by isolation, and these results were compatible with the blast results that revealed the existence of both with identity% = 96% and 98%, respectively. (Table 2.)

* 1. **Concordance between the conventional isolation and 16sRNA sequence:**

Three samples have elaborated a complete concordance in the results of both diagnostic tests; as shown in table 2, samples 1 and 4 were positive for E.coli, while sample 5 was positive for both E.coli and Salmonella either by isolation or 16s RNA sequence. On the other hand, the rest seven samples have a partial concordance, as the isolation was selective for only one bacterial species while the 16s RNA sequence revealed more than one causative agent.

**Table (1 ) results of conventional culturing techniques of examined samples**

|  |  |
| --- | --- |
| **Incidence of *Salmonella***  | **4 /10 (40%)** |
| **Incidence of E.coli** | **7/10 (70%)** |
| **Incidence of *Klebsiella***  | **1 /10 (10%)** |

Table (2): Identity percent rates between clinical and 16S rRNA-based identification.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | samples of Clinical Identification | Clinical Identification results  | 16S rRNA Identification results | Identity %  |
| 1 | Heart | E. coli | *E. coli*  | (92%) |
| 2 | Liver  | Salmonella  | *Salmonella enterica*  | (94%-96%) |
| *E. coli* | (96.8%) |
| 3 | liver | SalmonellaKlebsiella | *Enterobacter spp* | (99%) |
| *E. coli*  | 97% |
| *Salmonella spp*  | (96%-99%) |
| *Klebsiella spp*  | (97-98%) |
| 4 | liver | E. coli | *E. coli*  | (97%) |
| 5 | liver | E. coliSalmonella | *E. coli*  | (95-96%) |
| *Salmonella*  |  (98%) |
| 6 | oviduct | E. coli | *E. coli*  | (99%) |
| *Salmonella*  |  (96%-98%) |
| 7 | oviduct | E. coli | *E. coli*  | (98%) |
| *Salmonella* |  (96%-98%) |
| 8 | liver | Salmonella | *E. coli*  | (97%) |
| *Salmonella enterica*  |  (94%-96%) |
| 9 | liver | E. coli | *E. coli*  | (97%) |
| *Salmonella enterica*  | (94%-96%) |
| 10 | liver | E coli | *E. coli*  | (99%) |
| *Salmonella*  |  (94%-96%) |

1. **Discussion**

In some studies, the conventional biochemical tests failed to differentiate between species of *E coli* and *Salmonella*. 16s RNA sequencing is a useful and practical solution for the identification of bacteria, particularly the unusual aerobic Gram-negative bacilli ( Woo et al. 2000).

Detection and examination of bacterial genome still lacking, despite of its value in tracing any change in the bacterial genome that traditional methods don't offer or support detection of these changes, moreover, it helps in detection of causative agents in outbreaks, epidemiological mapping, phylogenetic classification of bacteria (21), surveys ( Sundquist et al.,2007), surveys (Whiteley et al,2012), bacterial identification and taxonomy (Clarridge 2004, Mignard and Flandrois 2006)

Different bacterial agents may be hard to be identified through culturing methods or take a long time about 5-7 days until biochemical confirmation such as *Salmonella,* (Akbar and Anal, 2015), also differentiation is important due to the rapid progress of antimicrobial resistance, with *E coli* and *Salmonella* have shown MDR to many ordinarily used antimicrobials (Moawad et al.,2017). However, the 16 sRNA sequencing technique has avoided the artifacts of the culturing, it consumes less time than other traditional methods, with higher sensitivity (Mignard and Flandrois 2006))

Despite 16sRNA needs expensive supplies and equipment, also a well-trained lab personnel (Horton et al.,2018) it will be available in research labs more than in a diagnostic lab, but with the rapid development of diagnostic aids the cost of this technology won't be an obstacle by time ( Muhamad Rizal et al. 2020 ), this technology has been applied in US Food and Drug Administration Precision Medicine Initiative in oncology and genetics fields and aiming to be distributed for early detection of infectious diseases (Culbreath et al. 2019). Furthermore, the sustainable improvement of the new rapid and different techniques for antimicrobial susceptibility testing allows tracing bacterial growth within a few hours(Wistrand-Yuen et al. 2020). However, the time-saving prosperity of 16s RNA sequencing as a modern technique, regardless of the expense, assist in rapid interference and proper implementation to control the infection (Table 3)

**Table (3): comparison between the culturing and 16s RNA sequencing:**

|  |  |  |
| --- | --- | --- |
|  | **Culturing**  | **16s RNA sequencing** |
| Time-consuming | Longer (5-7 days) | Shorter (3-4 days) |
| sensitivity | Can detect the major bacteria species in samples  | Can detect all the bacteria in samples even in traces |
| Specificity  | Detect only one type of bacteria | Can differentiate between different types of bacteria  |
| Laboring and equipment | Feasible  | hared |
| Expense  | lower | higher |

In this study, the nucleotide sequence in each sample has been identified by the BLASTn tool in GenBank (NCBI). However, the nucleotide sequences in some samples were similar to more than one bacterium species belonging to the family Enterobacteriaceae, contrary to the results of conventional isolation that revealed only one species in each sample. Sometimes, the isolation technique results in one type of suspected bacteria but the nucleotide sequence of the 16S rRNA could be related to more than one bacterium ( Jenkins et al. 2012). As the 16S rRNA gene is universal, more than one type of bacterium could be detected in the same samples (Maskell et al. 2006; Schabereiter‐Gurtner et al. 2008). For example, it was not possible to distinguish between the 16S rRNA sequences of *E. coli* and *Shigella* sp (Jenkins et al. 2012 ).

In this study, we have examined ten chicken flocks that suffered from signs of enteric infection as greenish diarrhea with an offensive odder. We have collected the internal organs other than gastrointestinal organs to detect the systemic circularization of the suspected bacteria. Accordingly, the suspected enteric bacteria have been isolated and detected in different organs, the determined bacteria have been confirmed depending on the case history of the suspected flocks. Multiple enteric bacterial genomes have been determined by the 16s RNA sequencing in some cases, so these results have pointed to the accuracy 16s rRNA technique. As we aforementioned, 16s RNA sequencing can detect multiple causative bacteria. The concordance between the results of 16s RNA sequencing and the traditional culturing, besides the case history, confirms the accuracy of the determined bacterial genome by the 16s RNA technique. Additionally, these findings have supported the sensitivity of the 16s to detect all the copies of the suspected causative bacteria that could not be cultured and proved the specificity of 16s to differentiate between the different suspected bacteria in the examined samples(Salipante et al. 2013).

There are nine variable regions (v1-v9) on 16s rRNA that can be used to discriminate between diﬀerent bacterial species (Chakravorty et al. 2007). However, no single region can be used to diﬀerentiate among all known bacteria. Therefore, we used primers to cover a big part of the 16S rRNA gene to assist in elevating the ability of discrimination, although the sensitivity may be decreased ( Sune et al. 2020 ). the sensitivity of the assay is affected by the size of the 16S rRNA gene fragment amplified ( Jenkins et al. 2012 ).

Failure of the PCR methods to detect the 16S rRNA gene in culture-positive samples may be due to the number of bacteria present in a sample being lower than the detection limit of the method; also PCR inhibitors may have been present in the sample (Harris and Hartley 2003; Schuurman et al. 2004).

Sanger sequencing of the 16S gene is limited to detecting two or

perhaps three species in the clinical samples (Kommedal, et al., 2008). Therefore, it would rather apply the next-generation sequencing to get a higher

resolution ( Stavnsbjerg et al. 2017 ).

1. **Conclusion**

16S RNA Sequencing was found to have better sensitivity results than the culturing techniques, so it is important to detect causative agents in different samples, but must be accompanied by complete case history. moreover, it is recommended to use 16S RNA Sequencing, especially in case of negative culture results accompanied by a diseased picture

1. **Authors’ contributions**

All authors contributed to the conduction, writing, and review of the manuscript. The authors read and approved the final manuscript.

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1. **Declarations**
2. **Competing interests**

 The authors declare that they have no competing interests.

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