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Article

Comparative *In Vitro* Susceptibility of *Proteus* Species Isolated from Clinical Specimens to Honey, Ciprofloxacin and Tetracycline

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Abstract: Comparative *in vitro* susceptibility of *Proteus* species isolated from clinical specimens to ciprofloxacin, tetracycline and honey was evaluated using standard microbiological techniques. Honey was diluted by percent volume per volume concentration of 100%(neat), 80%(v/v), 60%(v/v), 40% (v/v), 20% (v/v) and 10% (v/v) and its antimicrobial activity against *Proteus* species compared to ciprofloxacin and tetracycline. A honey concentration of 100% (neat) had the highest effect on *Proteus* species with a mean zone of inhibition of 28mm. Concentrations of 80%(v/v), 60%(v/v), 40% (v/v), and 20% yielded mean inhibition zones of 26mm, 23mm, 20mm, and 15mm respectively. Only four isolates gave inhibition zones at 20%(v/v) dilution. Tetracycline and ciprofloxacin inhibited *Proteus* species with comparatively lower mean zones of inhibition (15mm for tetracycline and 16mm for ciprofloxacin). The minimum inhibitory concentration (MIC) of honey for 86.7% of the isolates was 40%(v/v) and for the remaining was 20%(v/v). The MBC values were the same for their MIC. This study reveals a significant level of susceptibility of *Proteus* species isolated from clinical specimens to honey, tetracycline and ciprofloxacin, with undiluted honey exhibiting comparatively higher zones of inhibition. It also revealed a high level of resistance to tetracycline by *Proteus* species isolated from automobile wounds. Further studies using uniform concentrations of the honey and the evaluated antibiotics may be necessary before honey may be considered a better treatment option.

Key words: honey, *Proteus*, ciprofloxacin, tetracycline, MIC, MBC.

1. Introduction

Honey, which is produced from many different floral sources by bees, is an ancient remedy for the treatment of various ailments. Its application for the treatment of wounds dates back to hundreds of years ago, even before the discovery of bacteria as causes of infections. Dioscorides in 50 A.D documented its recognition for effectiveness against rotten and hollow ulcers (Molan, 1992). Modern interests in its applications and uses are increasingly gaining popularity and acceptability globally following reports of its successful uses, most especially where conventional antibiotics have failed (Efem *et al*., 1992; Mboto *et al*., 2009).

Some laboratory-based studies have revealed that honey promotes healing, have antimicrobial action against a broad spectrum of bacteria and fungi including methicillin resistant *Staphylococcus aureus*(MRSA) and other antibiotic-resistant bacteria (Cooper *et al*., 1999).

Generally, *Proteus* species are among the commonly implicated pathogens in hospital as well as community acquired infection (Douglas *et al*., 2000). The pathogen has a diverse mode of transmission, and hence can cause infection in different anatomical sites of the body. Some of the incriminating sources of transmission are soil, contaminated water, food, equipment, intravenous solutions, the hands of patients and health care personnel (Emori and Coyness, 1993; Heinzelmann, 2002).

In many resources limited countries including Nigeria, bacteriologic diagnosis up to the identification of the species are not common due to the cost and the special skills involved (Ohene, 1997; Newman, 2011), hence, there is limited documented information relating to patient demographics and antibiotic susceptibility levels for infections caused by the various species of *Proteus*.

This study is therefore aimed at determining the susceptibility of *Proteus* species isolated from clinical specimens to honey in comparison to ciprofloxacin and tetracycline, which are the common antibiotics of choice for the treatments of most infections caused by *Proteus* species.

2. Materials and Methods

2.1. Study Site

This experimental study was conducted in the laboratory of the Department of Microbiology, University of Uyo, Uyo from March 16- May 10, 2011.

2.2. Source of Honey and Validation of Its Purity

Pure natural honey harvested from the wild was purchased from a reputable dealer in Obudu, Cross River State of Nigeria and dispensed into sterile screwed cups. Fresh, dark and unadulterated were the criteria for purchase. In the laboratory, its purity and confirmation of non-adulteration was determined through physical examination for color, viscosity, non-infected with insects. In addition, its non-adulteration with water, and production of igniting a blue flame in light and the split fire test were mandatory criteria for acceptability. Finally, the honey was filtered with a sterile mesh to remove debris and then streaked on blood agar and Sabouraud plates, and incubated at 37 ºC overnight. Each plate was examined in turn for absence of microbial contaminants. The honey was then stored refrigerated (2-8 ºC) until required for use.

2.3. Source of Proteus Isolates

Proteus species isolated from clinical specimens were obtained from the Microbiology Departments of the University of Uyo Teaching Hospital, Uyo and University of Uyo Medical Centre. Close to sixty percent (60%) of the isolates were from automobile wounds, twenty six percent (26%) from urine samples (predominantly catheterized specimens of urine-CSU) and the remaining (13.3%) from miscellaneous sources (ear swabs, pus aspirates etc.). Their identification was further confirmed using standard microbiological techniques and identification characteristics as described in Cowan & Steel's Manual for the Identification of Medical bacteria (Barrow and Fulton, 2003). Each isolate was maintained in the laboratory on nutrient agar slopes at 4 ºC. A summary of the sources of isolates is presented in table 1.

Table 1: Sources of Proteus isolates

Clinical specimen	**No of Specimen (%)** (n=30, %)
*CSU	6 (20)
Other Urine samples	2 (6.6)
Wound	18 (60)
Others (Cervical Smear, Ear swab etc)	4 (13.3)

*CSU- Catheterized urine specimen

2.3.1. Source of antibiotics and its preparation

Exactly 50µg/mL of Tetracycline (Tetracap, Fidson Drugs Nig. Ltd) was prepared from its 250 mg capsule using sterile distilled water and 2 mg/mL (ampoule) (0.2%) of Fidson Drugs Nig. Ltd. brand of ciprofloxacin (Cifran) was the antibiotics employed.

2.4. Control Standard Proteus Species

A freeze-dried culture of *Proteus mirabilis* (NCTC 10975) was obtained from the Microbiology Research unit of the Community Health Organization, Lambata and employed as a representative strain for the comparison of performance of clinical isolates of the *Proteus* species evaluated in this study. The freeze-dried culture was reconstituted in Trypticase Soy broth (Merck 1.05459) according to the instructions supplied, and incubated at 37ºC for overnight. It was sub-cultured onto nutrient agar plates and slopes and maintained in the laboratory at 4ºC until required for use.

2.4.1. Proximate analysis of honey

The recommended method of the Association of Official Analytical Chemist (AOAC, 1999) was employed to analyze proximate values of the honey. Moisture, ash, fiber, protein, and (fat) lipid contents were all determined, while its carbohydrate content was obtained by subtracting the sum of protein, fat, ash and fiber from the total dry matter. Its pH was measured using a pH meter (JEN WAY 3320).

2.5. Susceptibility of Proteus Isolates to Ciprofloxacin and Tetracycline

The susceptibility of each *Proteus* isolates to ciprofloxacin and tetracycline was done following the standard agar disc diffusion method (NCCLS, 1999). Briefly Muller-Hinton Agar plates (DST) were seeded with overnight broth cultures of each clinical isolate of *Proteus* species on a separate plate. Each isolate was inoculated in triplicate. The procedure was repeated for the standard control strain of *Proteus mirabilis* (NCTC 10975). Ciprofloxacin and tetracycline discs were aseptically placed in pairs on the surface of the plates and incubated for 24 hours at 37ºC. Susceptibility was determined on the basis of inhibition zones following the methods of recommended by NCCLS (2008). The mean inhibition zone of each isolate was then calculated.

2.6. Susceptibility of Proteus to Honey

This was carried out using a modified method of Nzeako and Hamdi, (2000). The honey sample was diluted to 100% (v/v), 80% (v/v), 60% (v/v), 40% (v/v), 20% (v/v) and 10% (v/v) of its

original concentration using sterile nutrient broth. Each dilution was done in triplicates. Muller – Hinton agar plates were seeded with the overnight broth culture of each *Proteus* isolate on a separate plate culture. The procedure was repeated using a standard control strain of *Proteus mirabilis* (NCTC 10975). Seven standard wells equidistant from each were aseptically bored on each seeded plate using a sterile cork borer. The seventh well was at the center of each plate and served as a control. Fifty micro liters (50µl) of each honey dilution were introduced into each well. The control well contained only 50µl of nutrient broth. Furthermore, sterility of the nutrient broth was ascertained by inoculation onto MacConkey, blood and Sabouraud agar plates. The plates were incubated at 37ºC for 24hours after which inhibition zones were measured in millimeters (mm) and average of the inhibition zones recorded following the methods of NCCLS (NCCLS, 2008).

2.7. Determination of Minimum Inhibitory Concentration (MIC)

Determination of MIC was based on the agar dilution method. To this end 40% (v/v) dilution of the honey was further diluted using nutrient broth to provide dilution values of 30% (v/v), to 10% (v/v) (4 tubes). Each tube was seeded with a loopful of overnight broth culture of the test organism and incubated for 24 hours at 37 ºC. The lowest concentration which showed no turbidity after the incubation period was regarded as the minimum inhibitory concentration.

2.8. Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration of the honey was determined by sub- culturing the broth dilutions of the honey without visible turbidity. The lowest concentration that yields no growth on the sub-culture agar plate after incubation for 24hours at 37 ^{o}C was regarded as the minimum bactericidal concentration.

3. Results

3.1. Proximate Analysis of Honey

The moisture and ash contents of the honey sample were 10.38% and 0.27% respectively. Its fiber content was 0.13%, protein content is 1.05%, fat and lipid content is 1.82% and carbohydrate content is 96.73%. Its pH was determined as 5.08. The summary of this result is presented in tables 2 and 3.

3.2. Susceptibility of Proteus Species to Honey

The results obtained from the *in vitro* susceptibility of *Proteus* species to honey were almost the same neglecting the source of the isolate. All the thirty *Proteus* species were sensitive to honey at neat and 86.7 % (26/30) to 40%(v/v) dilution and 13.3 % (4/30) to 20%(v/v) dilution of honey. The control strain was also sensitive at a concentration of 20%(v/v) of honey. No inhibition zones were produced by all *Proteus* isolates and control strain at 10%(v/v) dilution of the honey.

The zones of inhibition produced varied according to the source of clinical specimen the isolate was made. However, these differences were not significant ($p>0.05$). Similarly, the inhibition zones were directly proportional to the concentration of honey employed. Thus honey concentration of 100% (undiluted) gave a mean zone of inhibition of 28mm, 80%(v/v), 60%(v/v), 40%(v/v) and 20%(v/v) gave a mean inhibition zone of 26mm, 23mm 20mm and 15mm respectively. Similarly, the main zones of inhibition of the control strain were also proportional to the concentration of the honey employed. Undiluted (100%) gave a mean inhibitory zone of 31mm. Honey diluted to 80%(v/v), 60%(v/v), 40%(v/v) and 20%(v/v) gave a mean inhibition zone of 30mm, 27mm 25mm and 16mm respectively.

Table 2: Preliminary Test for Validity of Honey

Test	**Observation**
Color	Dark brown in color
Viscosity	Highly viscous
Split fire test	Ignite blue flame in light
Adulteration with water	Non- infected with insects

Table 3: Proximate Analysis of Honey

Parameter's	**Value (%)**
Moisture content	10.38
Ash content	0.27
Fiber content	0.13
Protein content	1.05
(Fat) lipid content	1.82
Carbohydrate content	96.73
pH	5.08

3.3. Susceptibility to Ciprofloxacin and Tetracycline

Like honey, all the *Proteus* isolates including the control strain were susceptible to 0.2% ciprofloxacin, (2 mg/mL) of ciprofloxacin. The mean zones of inhibition for all the isolates were 16mm, while the control stain gave a mean inhibitory zone of 19mm. However, unlike honey and ciprofloxacin, only twenty-five out of the thirty (83.3%) *Proteus* isolates evaluated in this study were susceptible to 50µg of tetracycline with a mean inhibition zone of 15mm. The control strain was susceptible to 50µg of tetracycline giving a mean zone of inhibition of 22mm. The five isolates of *Proteus* species that were resistant to tetracycline were all isolated from automobile wounds. A summary of this is provided in table 4.

Table 4: Antibacterial Activities of Different Concentrations of Honey Compared to Ciprofloxacin and Tetracycline

Parameter Evaluated (%/Concentration)	Mean Zones of Inhibition (mm)
Honey (100%)	28
Honey (80%)	26
Honey (60%)	23
***Honey (40%) (26 isolates)	20
**Honey (20%) (4 isolates)	15
Honey (10%)	0
*Tetracycline (50µg /ml) (83.3%)	16
Ciprofloxacin (2mg/mL)	15

*Susceptibility was by 83.3% of the isolates
** Susceptibility was by 13.3% of the isolates
*** Susceptibility was by 86.7% of the isolates

3.4. Minimum Inhibitory and Bactericidal Concentration Honey

The minimum inhibitory concentration (MIC) for 86.7% (26/30) of the isolates was 40% (v/v) and the remaining 13.3 % (4/30) was 20% (v/v). The MBC for the isolates were the same with their MIC.

4. Discussion

Honey produced by honey bees (Apismellifera) is one of the oldest traditional medicines known to man for therapeutic and prophylactic uses (Mulu*et al*., 2004, Mboto *et al*., 2009). The determined proximate composition of the honey employed for this study agrees with most similar studies that have evaluated the composition of honey (Molan, 1992; Dimitrova *et al*., 2007)

The finding in this study of a high level of susceptibility of all *Proteus* isolates neglecting its anatomical site of isolation to honey is in conformity with earlier reports of its efficacy against a wide range of both Gram positive and Gram negative organisms (Molan, 1992; Nzeako and Hamdi, 2000 and moto *et al*., 2009).

Molan (1992) more than two decades ago had revealed that the activity of honey against some important nosocomial agents including methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas* species. Similarly, Mboto*et al*., (2009) has also demonstrated that warm water extracts of the leaves of *Garcinia kola* and *Vernoniaamygdalina*suspended in honey exhibited significant antimicrobial activity against *Escherichia coli, Proteus mirabilis, Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiellapneumoniae*and *Candida albicans*. The results of our study are therefore consistent with some of these documented studies.

In this study, ciprofloxacin and tetracycline, two common drugs of choice for the treatment of infections due to a wide range of bacterial infections including *Proteus* species persistently produced comparatively smaller zones of inhibition as against the zones of inhibition exhibited by honey. One major reason for the comparative larger zones of inhibition shown by honey may be due to the comparative higher concentration at which honey was used. Thus the undiluted honey yielded almost twice the mean zones of inhibition showed by tetracycline or ciprofloxacin. This study reveals that honey when diluted to the same concentration with the antibiotics evaluated exhibited little or no antibacterial activity. However, its superior efficacy is exhibited when used in its natural state (undiluted) as it is traditionally applied. The finding that undiluted honey had the highest inhibition zone to *Proteus* is in agreement with the works of Efem*et al*., (1992) and Agbaje*et al*., (2006).

The standard control strain of *Proteus mirabilis* (NCTC 10975) employed as a representative strain for the comparism of performance of clinical isolates of the *Proteus* species evaluated in this study was approximately 150% more susceptible to honey concentrations of 100%, 80%, 60%, 40%, 20%n and to ciprofloxacin (2mg /ml) and tetracycline (50µg/mL). This finding further confirms the antimicrobial activity of honey.

Studies have revealed that a vast of the antimicrobial activity of honey may be due to its super-saturated sugar which exerts an osmotic pressure that makes little or no water available for microorganisms to survive. Thus, the dilutions of honey increase its water content and reduce its

osmolarity. This reduced osmolarity may provide an explanation for the comparatively lower antimicrobial activity of diluted honey found in this study.

In this study, the antibiotics ciprofloxacin exhibited a comparative superior activity against all isolates of *Proteus* evaluated as compared to tetracycline. This finding may not be unconnected with the local practice of misuse of common antibiotics such as tetracycline (Levy, 1999) that are comparatively cheaper.

Molan and Russel (1988) stated that the antimicrobial properties of honey may also be as a result of non – peroxide components which include complex phenols and organic acids often referred to as flavonoids. In this study, the phytochemical components of the honey employed was never evaluated, however, several studies have shown dark colored honeys contained more phenolic acid derivatives but less flavonoids than light colored ones (Amiot*et al*., 1989).

Honey used in this work gave variations in zones of inhibition that was almost proportional to its concentrations. This finding is in conformity with many reported investigations of the antimicrobial activities of honey (Bhat, 1998; Mohammed *et. al*., 2008).

Other *in vitro* studies have revealed that honey stimulate monocytes in cell culture to release cytokines, tumor necrosis factor (TNF) – alpha, Interleukin (1L-1) and 1L–6 which stimulate the immune response to infection (Tonks *et al*., 2001). This report is therefore suggestive that honey when used in *invivo* may produce a greater effect than the *in vitro* study, thus justifying is vast ethno-medicinal uses.

It appears certain that the source of isolation of the *Proteus* species may influence its susceptibility. In this study, comparatively larger zones of inhibition were associated with *Proteus* isolates from urine samples. Similarly, all the five isolates of *Proteus* species that were resistant to tetracycline were isolated from automobile wounds. These differences though not significant ($p>0.05$), cannot be justifiably explained within the scope of this work. However, Bhat (1998) observed that Gram negative isolates from wound samples were more resistant to honey than Gram negative organisms. Similarly Efem *et al.*, (1992) found significant disparities in susceptibility to honey between clinical isolates.

In a study reported by Bhat (1998), the minimum inhibitory concentration (MIC) of honey was 40 percent against Gram negative bacteria including *Proteus mirabilis* isolated from wound infections. The finding in this study of 86.7% (25/30) of the isolates with an MIC of 40% (v/v) is totally in agreement with this report. Some studies have however revealed that the type of honey, the source of the nectars (Akortha, 2007), its phenolic acid derivatives and flavonoids can influence their antimicrobial activities (Amiot *et al*, 1989). These factors were not evaluated in this study.

5. Conclusion

This study reveals a significant level of susceptibility of *Proteus* species isolated from clinical specimens to honey, tetracycline and ciprofloxacin, with undiluted honey exhibiting comparatively higher zones of inhibition. It also revealed a high level of resistance to tetracycline by *Proteus* species isolated from automobile wounds. Further studies using uniform concentrations of the honey and the evaluated antibiotics may be necessary before honey may be considered a better treatment option.

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