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Determination the site of antibiotic resistance genes in *Escherichia coli* isolated From Urinary Tract Infection

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Abstract: ***This study includes isolation of 25 isolates of Escherichia coli (E. coli) strain from urinary tract samples in a pregnant woman. Microbiological and biochemical tests were used to identify the resistant bacteria of this genus. Screening methods were used to determine bacterial isolates for their resistance to 10 antibiotics include: Amikacin (Ak), Amoxicillin (Ax), Ampicillin (Ap), Chloramphenicol (Cm), Ciprofloxacin (Cip), Erythromycin (Er), Nalidixic acid (Nal), Penicillin (Pen), Tetracycline (Tet) and Trimethoprim (Tm). The isolates E4, E9, E16, and E17 were resistant to all antibiotics used in the current study using the disk diffusion method. In contrast, the resistance percentage for all antibiotics ranged between 28-96%. Sites of resistance genes and hemolysin production genes were characterized by tranformation techniques in the E4 and E16. The results showed that the antibiotic resistance genes of Amikacin, Erythromycin, Tetracyclin, and Trimethoprim were located on a plasmid, whereas Amoxicillin, Ampicillin, Chloramphenicol, Ciprofloxacin, Nalidixic acid and Penicillin were located on chromosomal DNA. The results also demonstrated an inability to produce alpha or beta-hemolysin indicating that the genes which are responsible for hemolysin production were also located on chromosomal DNA.***

Keywords: *E. coli* , antibiotics, resistant genes, plasmid

1. INTRODUCTION

Urinary tract infections (UTIs) are defined as the presence of bacteria in urine along with symptoms of infection. The infection is important clinically because of many organelles of the urinary tract may involve such as urethra, bladder, uterus, and kidney. All age groups are susceptible to this type of infection, but the frequency in women is higher than men [1] add reference??. The reason may be attributed to many factors including short urethra, the absence of prostates secretion, pregnancy and easy contamination of the urinary tract with fecal flora. Additionally, during pregnancy, the physiological increase in plasma volume decreases the concentration of urine up to 70%. As a result, pregnant women develop glucosuria, which encourages bacterial growth in the urine [2].

Antibiotic resistance microorganisms are a health threat in the world which encourages the medicine manufactures to produce new generations of yearly antibiotics to solve this problem, especially there are some bacteria considered a multiple antibiotic resistance such as *Escherichia coli*, *Klebsiella spp*., *Staphylococcus spp.*, *Pseudomonas spp.*, *Proteus spp.* and others [3].

One of the major problems that associated with the risk of *E. coli* is their ability to resist a wide variety of antibiotics that introduced as therapeutic agents. These mainly due to the presence of extrachromosomal elements that harbor a resistant gene or R-factor which can be passed among bacteria of the same species or to other strains of *E. coli* through different mechanisms include transformation, conjugation and transduction processes. Therefore, controlling and elimination of the resistance that conferred by R-plasmid by using biological mutagens, physical and chemical or other curing agents will be quite useful to put out such resistance eliments [4].

Finally, the epidemiology of drug resistant *E. coli* and other gram-negative enteric bacteria is undergoing a remarkable change in the feature with the widespread occurrence or resistance transfer factors (RTF), which may have transferred to drug-sensitive strain by conjugation and transduction [5]. So the present work comprises: Isolation and identification of *E. coli that* cause urinary tract infection in pregnant women, study the resistance of *E. coli toward* different antibiotics understudy, isolation and purification of plasmid DNA content from *E. coli*, and determination the site of antibiotic resistance genes by genetic transformation.

2. METHODS AND MATERIALS

2.1 Specimens collection

Urine sample: Mid-stream of 75 urine samples were collected in sterile cups from pregnant women suffering from urinary tract infections attending Health Center (Nazdar Bamerni), during the period 15th July 2017 to 7th September 2017.

2.2 Processing of Samples:

2.2.1 General urine examination:
For microscopic examination, 5ml of urine was centrifuged, the supernatant was discarded, the deposit then re-suspended, and 0.1ml applied on a clean microscopic slide. The precipitated material was examined for the presence of leukocytes (pus cells), erythrocytes (RBC), mucus, bacteria, and parasites, along with crysals and casts[6].

2.2.2 Urine culture (isolation of *E. coli)*
A loop full of undiluted urine samples was spread on culture media (blood and MacConkey agar) plates. The plates were incubated at overnight at 37°C. Well isolated single colonies were sub-cultured on the same media to check for the purity of the isolated bacteria. Purified isolates were identified using microscopic, morphological, biochemical and API 20E system as a more accurate method for identification

2.3 Hemolysis on blood agar
On blood agar, *E. coli and* most of the other enteric bacteria form circular, convex, and smooth colonies with B-hemolysis produced by some strains of *E. coli* [4].

2.4 Antimicrobial susceptibility test
This test was performed according to write the name of the author with no year then [6]. Antibiotic-impregnated discs with required concentration were dispensed on the surface of Mueller-Hinton agar medium that has been spread with a pure bacterial suspension of 105 CFU/ml. After incubation, inhibition zones were measured and translated into predetermined categories as susceptible, intermediate, or resistant (Table-3).

2.5 Isolation of plasmid DNA content from bacterial isolates understudy
In order to isolate the plasmid DNA, a method described by [7] was employed with some modification and includes the following steps: 50 ml of sterilized nutrient broth media containing appropriate antibiotic was inoculated with single colony of tested bacterial isolate, and incubated in a shaking incubator (100 rpm) at 37°C overnight. Then, centrifugation was carried out for 10 minutes at 8000 rpm. The pellet re-suspended in 2 ml solution I (Glucose 20%, EDTA 20Mm and Sodium Dodecyl Sulfate 10%), then 100µl of 50µg /ml lysozyme (BHD, England) was added to the suspension, left at room temperature for 10 minutes, after that 4ml of solution II added, vortexed and left on ice for 10 minutes. Then 2 ml of cold 5M potassium acetate was added and left on ice for 10 minutes, after that centrifugation at 8000 rpm to precipitate cell debris and high molecular weight DNA were performed. In order to remove protein, the supernatant transferred to clean tube and an equal volume of chloroform: Isoamyl alcohol (24:1) was added, centrifuged for 10 minutes at 8000 rpm, this step repeated several times. The aqueous layer was transferred to clean tube and its volume estimated, then 1/10 of this volume solution III added with two volume of absolute ethanol to precipitate the nucleic acids, the mixture was left for 30 minutes at -20°C, then centrifuged for 10 minutes at 8000 rpm. The pellet washed with 5 ml of 70% ethanol, centrifuged for 10 minutes at 8000 rpm, after that the pellet dried, re-suspended with 0.5 ml Tris-EDTA buffer and stored at -20°C.

2.6 Spectrophotometric quantification of plasmid DNA
A method utilized by Sambrook and Russel [8] was followed: A 100 microliter of prepared plasmid DNA was diluted by TE buffer to 1ml and the optical density (O.D.)was measured at 260 nm. The concentration of plasmid DNA calculated according to the equation:

Optical density of DNA X dilution factor X 50µg/ml=No.µg/ml

2.7 Site determination of the genes resistance to the antibiotic
The procedure carried out by isolation and purification of DNA plasmid from the bacterial isolates. The DNA was transformed into standard strains of *E. coli which* used as bacterial hosts for DNA uptake. The transformation process of the isolated DNA plasmids was performed as follow:

2.7.1 Preparation of the competent cells
Competent cells were prepared using 5 ml of Lactose broth which was inoculated with a single colony of *E. coli DH5α* free of plasmids. The samples were incubated in a shaker incubator (100 rpm) for 24 hours at 37ºC and then one ml of bacterial culture was added to 50 ml of LB broth and the samples were incubated with shaking at 37°C for 3-4 hrs. The optical density of the bacterial cultures growth (OD650) was taken using a spectrophotometer to measure the logarithmic-phase of *E. coli* which typically ha an (OD650 of 0.5–0.7). The cells were harvested by centrifugation at 5,000 rpm for 10 min and the supernatant was discarded, then the pellet was resuspended one ml of ice-cold 0.1M CaCl2. The same solutions and concentrations were used for all the samples. The resuspended cells samples were left on ice for 30 min followed by centrifugation for 10 minutes with the same above conditions. Finally, the supernatant discarded, and the pellet of bacterial cells was resuspended in 2.5 ml of ice-cold CaCl2 [8].

2.7.2 DNA uptake
Transformation of DNA plasmid to competent cells of *E. coli* DH5 α:
Extracted DNA plasmids from isolate strains of *E. coli* were plated (Ten µl) on LB agar and incubated at 37ºC for 24 hours to ensure that the DNA plasmids are free from bacterial cells. Five µl of DNA plasmid was added to a

sterile Eppendorf tube contained 200 µl competent cells of *E. coli DH5α*. Negative controls which consisted of competent cells without DNA for each transformation samples were plated on LB agar supplemented with certain antibiotics. The mixtures were placed on ice for 30 min and then incubated in a 42°C water bath for 90 seconds. The transformation samples were cooled on ice for 5 min and transferred to 1.5 ml LB broth in tubes and incubated with shaking at 37ºC for 1 hour in a shaker incubator. The samples were transferred to 1.5 ml tubes for centrifugation at 14,000 r.p.m for 1 min. Finally, the supernatant was discarded from each sample and the pellets were spread on selective LB agar plates and incubated at 37ºC for 24 hours [9, 10].

2.7.3 Plasmid profile

The Tris-Borate Ethelyne Diamine Tetra Acidic acid (TBE) was used to prepare agarose gel (0.7%). This is in order to identify the bands of DNA through the gel electrophoresis as per their sizes. Melting 0.7 g of agarose gel powder in 100 ml of 1X TBE as a buffer by heating in a microwave for 2 minutes of boiling temperature. Then after, it has been cooled down in room temperature to 55ºC prior to pour into the gel electrophoresis tray containing the combs for DNA well and left for to be solidified. The gel and the tray filled with buffer of TBE 1X to sink the gel for 2-3 min, the combs removed carefully. As for molecular DNA marker, a ladder of 1 kb DNA (Fermentas) has been used. The wells were filled precisely with 10µl plasmid DNA extract previously mixed with 3µl of loading buffer. Voltage of 45 for 15 minutes was current to the system for 15 minutes, then after the voltage was set for a higher voltage (75 volts) and let to run for 90 minutes. The dye will be then noticed at the other pole of the gel. Soaking the gel in ethidium bromide (0.5 µg/ml of D.W.) was necessary for half an hour in order to visualize the gel by UV-trans illuminator, figure (8).

3. RESULTS AND DISCUSSION

3.1 Isolation of bacterial samples:

Table (1) recognizes the percentage occurrence of the bacterial isolates retrieved from 75 urine specimens. This came as in following order: *E. coli* (44.60 %), *Klebsiella pneumoniae* (17.80 %), *Staphylococcus spp*. (12.50%), *Proteus mirabilis* (12.50%), *Pseudomonas aeroginosa* (7.10 %), and *Enterobacter spp* .(5.30%) respectively. There is strong similarity between agents of microbes who cause UT infection whether the patient is pregnant or not. The organisms that cause UTIs during pregnancy are the same as those found in non-pregnant patients. The vast majority of infections (80%-90%) revealed *E. coli*.

3.2 Identification of *E. coli* isolates

The laboratory identification of *E. coli* isolates was carried out as per microscopical, cultural, morphological, and biochemical tests. Microscopic slide smear revealed short rods, motile, negatively stained with gram stain. They were motile and formed no spores. This led to a clear conclusion of the diagnostic feature of *Escherichia coli*, in accordance to other resources [14].

Table 1: Percentage occurrence of the bacterial isolates

Bacterial isolates	No.	Percentages %
E. coli	25	44.60
Klebsiella pneumonia	10	17.80
Staphylococcus spp.	7	12.50
Proteus mirabilis	7	12.50
Pseudomonas	4	7.10
Enterobacter spp.	3	5.30
E.coli	25	44.60
Klebsiella pneumonia	10	17.80
Staphylococcus spp.	7	12.50
Proteus mirabilis	7	12.50

According to their pink colony appearance on MacConkey agar as lactose fermenters, and grayish white moderately opaque with or without zone of hemolysis on blood agar, all *E. coli* isolates characterized by producing small, smooth, entire and convex colonies on blood agar. There was red pink lactose fermenting on MacConkey agar and producing bright metallic green sheen colonies on Eosine Methylene Blue (EMB) agar. As well as the results of biochemical tests which shown in table (2) demonstrate that all bacterial isolates understudy was positive for indole, methyle red, catalase tests, but it was negative for vogas-proskaur, citrate utilization, oxidase, no H2S production. Nevertheless, for all *E. coli* isolates, lactose and glucose fermentation were indicated by a yellow slant and a yellow butt A/A reaction On Kligler Iron Agar (KIA) medium [15].

Table 2: Some biochemical tests for identification of *E. coli*

IMVC test				KIA			Hemolysin			Others	
Ind	MR	VP	Cit	Gas	H_2S	Reaction	Œ	ß	δ	Oxidase	Catalase
+	+	–	–	+	–	A/A	10	2	13	–	+

The production of hemolysins alpha and beta by the uropathogenic *E. coli* can cause lysis of urinary tract cells. The results revealed that out of 25 *E. coli* isolates, 10 isolates were œ- hemolytic, 2 isolates were ß-hemolytic, and 13 isolates were gamma hemolytic representing 40%, 8%, and 52% respectively. Our results agree with those reported elsewhere [16, 17] where they found a large proportion of human extraintestinal *E. coli* isolates produce hemolysin representing (35-50%) and (35-60%) respectively, relative to normal fecal isolates (10%). Also they mentioned that epidemiological evidence indicates a role for alpha-hemolysin in extraintestinal human infections. In general, and according to Analytic Profile Index, the synonyms number of the retrieved specimens reveals that all isolates were *E. coli*, as shown in figure (1).

Figure 1: API 20E strip revealed E.coli (7144572)

3.3 Antibiotic resistance pattern for the bacterial isolates

The twenty-five *E. coli* isolated from urine samples and laboratory strain *E. coli* DH5œ were screened. This in order to indicate their level of resistance to ten widely used antibiotics depending on the final concentration of antibiotics (table 3). The patterns of the antibiotic resistance for the isolated bacteria are shown in (table 4). The percentages of resistant were quarantined and the isolates of the present study showed remarkable diversity in their resistance to used antibiotics used. The resistance rate of *E. coli* isolates ranged from 100%, for E4, E9, E16 and E17, to 50% For E15, E18 and E22, while the percentages of resistance for other isolates ranged between 60% and 90%. Table (5) revealed that the percentage of resistant bacterial isolates to different antibiotics.

Table 3: The standard inhibition zone for different antibiotics

Antimicrobial agent	Concentration (µg/ml)	Resistant mm or less	Intermediate mm	Sensitive mm or more
Amikacin (AK)	30	14	15-16	17
Ampicillin (AMP)	10	13	13-15	17
Amoxicllin	30	13	14-17	18
Erythromycin	25	10	11-15	16
Nalidixic acid (NA)	30	13	14-18	19
Tetracycline	300	14	15-16	17
Ciprofloxacin (CIP)	5	15	16-20	21
Penicillin	30	13	14-19	20
Chloramphenicol (C)	30	12	13-17	18
Trimethoprim	30	14	15-22	23

Table 4: Antibiotic resistance pattern for *E.coli* isolates understudy

Isolate No.	N. agar plates with final antibiotic concentratonis in µg/ml										
	AK	Ax	AP	Cm	Cip	ER	Nal	Te	Tm	Pen	Res.
E1	S	R	R	R	S	R	R	R	S	S	60%
E2	S	R	R	R	S	S	R	R	R	S	60%
E3	S	R	R	R	R	R	S	S	S	R	60%
E4	R	R	R	R	R	R	R	R	R	R	100%
E5	S	R	R	R	S	R	R	R	S	R	70%
E6	S	R	S	R	R	R	R	R	S	R	70%
E7	R	R	R	R	R	R	R	S	R	S	80%
E8	S	R	R	R	R	R	R	R	R	R	90%
E9	R	R	R	R	R	R	R	R	R	R	100%
E10	S	R	S	R	S	R	R	R	S	R	60%
E11	R	S	R	R	S	R	S	R	S	R	60%
E12	S	R	R	S	S	R	R	R	R	S	60%
E13	S	R	S	R	S	R	R	R	R	S	60%
E14	S	R	R	R	R	R	R	R	R	R	90%
E15	S	R	S	S	S	R	R	R	S	R	50%
E16	R	R	R	R	R	R	R	R	R	R	100%
E17	R	R	R	R	R	R	R	R	R	R	100%
E18	R	R	R	R	S	R	S	S	S	S	50%
E19	S	R	R	R	S	R	R	R	S	R	70%
E20	R	R	R	R	R	R	R	R	S	R	90%
E21	S	R	R	R	S	R	R	R	S	R	70%
E22	S	R	S	S	S	R	R	R	R	S	50%
E23	S	R	R	R	S	S	R	R	R	S	60%
E24	S	R	R	R	S	R	R	R	R	R	80%
E25	S	R	R	R	S	R	R	R	S	S	60%

R: resistance S: sensitive

Table (5) elucidated that the highest percentage of resistance was 96% to Ap, while the lowest percentage was 32% for Ak. The results also clarified that *E. coli* isolates showed different resistance to Ery, Tet, Cm, Nal, Ax, Pen, Tm and Cip and the resistant rate were 92%, 88%, 88%. 88%, 80%, 64%, 52%, and 40% respectively. Multiple resistances to two or more antibiotics was common. The results revealed that resistance to ampicilin and erythromycin was 96% and 92% respectively, similar results were reported [18, 19, and 20] they found that resistance of isolates of *E. coli* retrieved from urinary tract infections to ampicilin were 90%, 95% and 92% respectively. Studies elsewhere [21, 22] stated that no *E. coli* isolates from UTIs were sensitive to ampicillin.

Table (5): Percentages of resistant bacterial isolates to different antibiotics

Antibiotic	AK*	AX	Ap	Cm	Cip	Er	Nal	Pen	Te	Tm
No. of resistant	8	20	24	22	10	23	22	16	22	13
% Resistance	32	80	96	88	40	92	88	64	88	52

In the present study high resistance percentages (88%) was reported to chloramphenicol, tetracycline, naldixic acid. However, [23] found the resistant percentages of *E. coli* to naldixic acid were (86%), while [22] stated that a resistance percentage of *E. coli* to nalidixic acid was (20%). The resistance to quinolones as naldixic acid in gram- negative bacteria such as *E. coli* is mainly recalled to a mutation in their chromosome which modifies the targeted enzyme of the bacterium (DNA gyrase) and topoisomerase IV, or enhnce the efflux pump system that drain the drugs out of the cytoplasm.

This study showed that the resistant to tetracycline was (88%), similar results reported by [24], he also stated that resistant to tetracycline was (88%), resistant with a percentage of (79%) obtained in other study [22]. These results correlated with the study done by [25], who revealed that (73.3%) of *E. coli* isolated from UTIs were resistant to tetracycline. Chloramphenicol resistant percentages that recorded in this study was (88%), while others reported (70%) of *E. coli* susceptible to chloramphenicol [24]. Moreover research showed that *E. coli* isolated from UTIs were resistant to chloramphenicol at a percentage of (60%) [18], others reported that (39%) of *E. coli* isolates were resistant to chloramphenicol [26]. This diversity and multiple antibiotic resistance of *E. coli* are linked to genes located on their conjugant plasmid. Nevertheless, processes of conjugation, transformation or transduction can aid the transfer of these genes to recipient *E. coli.* The resistance genes can also occur on the circular chromosome of the bacterium and can dive to the plasmid by a process of transposition. Transposon may bear genes of multidrug resistance and both gram-positive and gram negative bacteria can perform such a process of transposition of genes [27].

3.4 Determination of the genetic site determination of antibiotic resistance in *E. coli*

The isolates of the present study were gone through a transformation experiment in order to uncover the locus of the antibiotic resistant gene in the *E. coli* isolates. Transformation was obtained by using DH5œ *E. coli* strain, as a recipient cell. It has the capability to resolve clean and purified *E. coli* plasmid DNA content from bacterial isolates. Four isolates were chosen from the bacterial isolates that show resistance to most antibiotics understudy. The antibiotic resistance pattern of those isolates and the laboratory DH5œ strains are shown in (table 6).

Table 6: Antibiotic resistance patterns of chosen acterial isolates used for transformation process

No.of isolates	N-agar with final antibiotic concentration in µg/ml									
	Ak*	Ax	Ap	Cm	Cip	Er	Nal	Pen	Tet	Tm
E4	S	R	R	R	R	R	R	R	R	R
E16	R	R	R	R	R	R	R	R	R	R
DH5œ	S	S	S	R	S	S	R	R	S	S

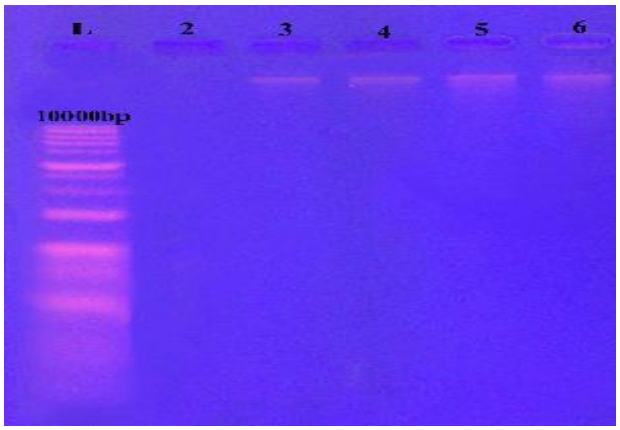
The L.B medium was used to subculture 10 colonies of DH5-α transformants. This process is crucial as it guarantees the phenotypic stability of resistance in the transformed colonies. In addition, it ensures proper and regular segregation of plasmids after purification. Finally, the colonies were tested for antibiotic resistance through Kirby-Bauer method. Results were as in table (7).

Table 7: Antibiotic resistance pattern after transformation of *E.coli* DH5-α With purified plasmid from *E.coli* isolates

No.of isolates	N-agar with a final antibiotic concentration in µg/ml									
	Ak*	Ax	Ap	Cm	Cip	Er	Nal	Pen	Tet	Tm
E4	S	R	R	R	R	R	R	R	R	R
DH5œ+ plasmid	S	S	S	R	S	R	R	R	R	R
E16	R	R	R	R	R	R	R	R	R	R
DH5œ+ plasmid	R	S	S	R	S	R	R	R	R	R

From the table, it's obvious that the transformation process performed successfully for each of E4 and E16. This is indicated by resistance to Er, Tm, and Te, for E4 transformant and to Ak, Er, and Tm for E16. The profile of the plasmids in transformed *E. coli* DH5- α strain with purified plasmid from E4, and E16 isolates (Figure 2) revealed that successful transformation is retrieved only in a single band (>10000 bp) that can show resistance of DH5-α to above antibiotics. This may be related to the fact that the responsible genes of a known resistance could be located on a large plasmid or the chromosomal DNA. This results in difficulty of uptake by the DH5œ receiver, or has a transposon property. Thus, we can conclude that all genes responsible for conferring resistance to antibiotics used (Ak, Er, Te, and Tm) are located on plasmid DNA.

Figure 2: Plasmid profile of transformant colonies



Lane 1 10 000 bp DNA ladder
Lane 2 *E. coli* DH5α laboratory strain
Lane 3 Plasmid content of E4
Lane 4 E4 transformant colonies
Lane 5 Plasmid content of E16
Lane 6 E16 transformant colonies

The size of the plasmids of bacterial isolates of which transformation test with DH5-α transformant performed can be the reason behind the diversity of antibiotics resistance. The size also determines the fluency and easiness off the entrance to the cells during transformation [28]. A study suggested that transferring f the genes horizontally may be a factor behind capability of clinical isolates to resist antibiotics [29]. Suggesting that consuming an antibiotic is not the only reason of the increased frequency of resistance to a particular antibiotic as previously stated by relevant works [30]. Research of others mentioned that after transformation with plasmid DNA in uropathogenic *E. coli* isolated from pregnant women, transformant colonies become resistance for gentamycin, trimethoprime, amikacin and tetracycline [31].

On the other hand, the ability of purified DH5œ transformant colonies to produce hemolysin were tested on blood agar using streaked plate method and the results demonstrate the inability of the transformant colonies to produce alpha or beta hemolysins [32].

4. CONCLUSION

From these results it is clear that the genes responsible for encoding hemolysin enzymes were located on chromosome and thus not transferred to the host competent cells during transformation.Alpha-hemolysin is a virulence-associated factor that has been found to be chromosomally encoded in most human urinary tract isolates and it has also been assumed that this determinant can be related to the pathogenesis of extraintestinal *E. coli* human infections.

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