Research Note

Presence and Correlation of Some Enteric Indicator Bacteria, Diarrheagenic Escherichia coli Pathotypes, and Salmonella Serotypes in Alfalfa Sprouts from Local Retail Markets in Pachuca, Mexico

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ABSTRACT

Data on the presence of diarrheagenic Escherichia coli pathotypes (DEPs) in alfalfa sprouts and correlations between the presence of coliform bacteria (CB), fecal coliforms (FC), E. coli, DEPs, and Salmonella in alfalfa sprouts are not available. The presence of and correlations between CB, FC, E. coli, DEPs, and Salmonella in alfalfa sprouts were determined. One hundred sprout samples were collected from retail markets in Pachuca, Hidalgo State, Mexico. The presence of indicator bacteria and Salmonella was determined using conventional culture procedures. DEPs were identified using two multiplex PCR procedures. One hundred percent of samples were positive for CB, 90% for FC, 84% for E. coli, 10% for DEPs, and 4% for Salmonella. The populations of CB ranged from 6.2 up to 8.6 log CFU/g. The FC and E. coli concentrations were between <3 and 1,100 most probable number (MPN)/g. The DEPs identified included enterotoxigenic E. coli (ETEC; 2%), enteropathogenic E. coli (EPEC; 3%), and Shiga toxin–producing E. coli (STEC; 5%). No E. coli O157:H7 strains were detected in any STEC-positive samples. In samples positive for DEPs, the concentrations ranged from 210 to 240 MPN/g for ETEC, 28 to 1,100 MPN/g for EPEC, and 3.6 to 460 MPN/g for STEC. The Salmonella isolates identified included Salmonella enterica serotype Typhimurium in three samples and Salmonella enterica serotype Enteritidis in one. STEC and Salmonella Typhimurium were identified together in one sample. Positive correlations were observed between FC and E. coli, between FC and DEPs, and between E. coli and DEPs. Negative correlations occurred between CB and DEPs and between CB and Salmonella. Neither FC nor E. coli correlated with Salmonella in the sprout samples. To our knowledge, this is the first report of ETEC, EPEC, and STEC isolated from alfalfa sprouts and the first report of correlations between different indicator groups versus DEPs and Salmonella.

The consumption of vegetable seed sprouts has been growing in global popularity over the past 10 years due to consumer demands for novel flavors and because consumers perceive health benefits of sprouts. However, a number of sprout-associated foodborne illness outbreaks have been reported since the 1990s, and Salmonella and Escherichia coli O157:H7 strains have been the most commonly associated pathogens (8, 19). Alfalfa and mung bean sprouts are most often implicated in outbreaks in North America (8, 19, 25). Sprout-related outbreaks of foodborne illnesses have also been reported in other parts of the world. An outbreak of E. coli O157:H7 infection from radish sprouts in Japan in 1996 led to 9,451 illnesses and 12 deaths (33). In Mexico, Salmonella has been isolated from raw alfalfa sprouts (6) and from raw mung bean sprouts (9). Sprouts are often eaten raw; this increases the potential infection risk associated with sprout consumption.

Diarrheagenic E. coli pathotypes (DEPs) (enterotoxigenic E. coli [ETEC], enteropathogenic E. coli [EPEC], Shiga toxin–producing E. coli [STEC], enteroinvasive E. coli [EIEC], enterogregarie E. coli [EAEC], diffuse adherent E. coli, and enteraggregative-hemorrhagic E. coli [EAHEC]), are important foodborne pathogens (3, 28). A recent foodborne outbreak of hemolytic-uremic syndrome caused by EAHEC O104:H4 in several European countries that involved sprouts highlights the importance of screening for DEPs in sprouts (3). This EAHEC outbreak involved more than 4,000 persons in 16 countries, and raw fenugreek sprouts were implicated as the vehicle of EAHEC. However the outbreak strain could not be identified on fenugreek seeds from the implicated lot (3). Greater frequencies of incidence data for DEPs are still needed for a wide variety of vegetables that are consumed raw, such as sprouts. In Mexico, DEPs have been associated with diarrheal illness both in native children (15, 16, 41) and in visitors to the country (32, 42). ETEC, EPEC, STEC, and enteroinvasive E. coli (EIEC) are important causes of diarrhea in developed
countries and in visitors from regions where DEPs are not endemic (15, 16, 35, 41). ETEC is the leading cause of
weanling diarrhea in developing countries and of traveler’s
diarrhea around the world (35). The predominant reservoirs
of ETEC are pigs and cattle (34). EPEC strains have been
isolated from a variety of animal species, such as cattle,
goats, sheep, chickens, pigeons, and gulls (12), and EPEC
strains have been responsible for outbreaks of diarrhea in
both developing and developed countries (35). STEC strains
are human pathogens associated with foodborne illness;
some strains can cause hemorrhagic colitis, which in some
cases may progress to hemolytic-uremic syndrome (35).
Ruminants, primarily cattle, are the predominant reservoir
of STEC (4). EIEC cause an invasive dysenteric form of
diarrhea in humans (35). Humans are a major reservoir for
EIEC (28). Foodborne bacteria, including DEPs, are usually
transmitted by contaminated food. Fecal contamination of
food and drinking water is the major route of infection by
pathogenic bacteria for humans (30).

The DEPs are classified based on their unique virulence
factors and can be identified by these traits alone. However,
each group has different pathogenic properties, different
virulence factors, different epidemiologies, and different
reservoirs (28, 35).

Recently, we isolated ETEC, EIEC, and STEC and
Salmonella from raw mung bean sprouts in Mexico (9);
however, with the exception of this study and with the excep-
tion of E. coli O157:H7 investigations in sprouts (8, 25, 33),
there are no reports in the relevant literature on the
presence of other DEPs in sprouted alfalfa seeds intended
for human consumption.

On the other hand, E. coli has been used as an indicator
of fecal contamination of food for many years (13). Numerous
studies correlate fecal coliform levels with the presence of E. coli. Nevertheless, the value of the fecal
coliform assay as a fecal contamination indicator is nullified
when nonfecal-origin bacteria are the principal microbes
detected by the assay (36, 38). It has been reported that
sprouts are generally contaminated with coliforms (6, 7, 9,
36, 44). These microorganisms are mainly nonfecal-origin
bacteria from seeds (44), and they increase in concentration
during the sprouting of seeds (7). In addition, Salmonella
strains have been isolated from sprout samples that tested
negative for E. coli (9). This means that FC and E. coli may
not be indicators of fecal pathogenic bacteria (such as DEPs
or Salmonella) on sprouts. The objective of this study was
to measure the presence of coliform bacteria (CB), fecal
coliforms (FC), E. coli, DEPs (ETEC, EPEC, EIEC, STEC,
EAEC, and diffuse adherent E. coli), and Salmonella on
alfalfa sprouts from public markets and to determine the
correlations between CB, FC, E. coli, DEPs, and Salmo-

MATERIALS AND METHODS

Sprout samples. A total of 100 alfalfa (Medicago sativa)
sprout samples (250 g each) were purchased in the four largest
public markets in Pachuca, Hidalgo State, Mexico. The city of
Pachuca has 13 public markets, but only 4 of these are sizable.
Sprout samples were collected from 5 vegetable retailers in each of
these 4 large markets (total of 20 retailers) during a 10-week study
period in the summer. Samples were purchased every 2 weeks at
each retailer, resulting in 20 samples per day and a total of 100
alfalfa sprout samples for the entire study period. At the time of
purchase, the sprouts were packaged in plastic containers and sold
at room temperature. The sprouts had been packaged in plastic
containers by the retailers or sprout producers. The plastic
containers containing sprouts were placed in sterilized plastic bags
in a cooler with frozen gel packs for transport to the laboratory and
analyzed no more than 1 h after purchase.

Sample preparation. Subsamples (100 g) from each sample
were placed in a sterile plastic bag, and 900 ml of sterilized lactose
broth (Bioxon, Becton Dickinson, Mexico) was added to each
sprout subsample. The subsamples were pummeled in a stomacher
(Seward, Worthing, UK) at 260 rpm for 1 min, and sample
dilutions for microorganism counts were prepared using sterile
tepone water (0.1%). Subsamples were analyzed to detect the
presence of CB, FC, E. coli, Salmonella, ETEC, EPEC, EIEC,
STEC, and EAEC as previously described (9, 23).

Microbiological analyses. CB, FC, and E. coli were
determined exactly as described in the U.S. Food and Drug
Administration bacteriological analytical manual (BAM) (48).
Briefly, for CB, 1 ml of each dilution was transferred to petri
dishes, and violet red bile agar was then poured into each plate,
followed by incubation at 35°C for 48 h. FC and E. coli were
analyzed by the most-probable-number (MPN) procedure: 1 ml of
serial dilutions in 0.1% sterile peptone water of each subsample
homogenate was inoculated into nine tubes containing lactose
broth (Bioxon) (three tubes from dilution 10⁻¹, three tubes from
dilution 10⁻², and three tubes from dilution 10⁻³) and into
Durham tubes. After incubation at 37°C for 48 h, a loopful of
positive culture suspension (determined by turbidity and gas
production) was transferred to tubes containing fluorocult brilliant
green 2%--bile lactose broth (Merck, Darmstadt, Germany). After
incubation at 44.5 ± 0.2°C for 48 h, tubes that were positive for
growth and gas production were considered positive for FC. For
determining the presence of E. coli specifically, FC-positive tubes
were used to identify indole formation (48). All tubes that were
positive for indole and gas production were streaked onto eosin
methylene blue agar (Bioxon). Two to three presumptive E. coli
colonies were selected from methylene blue agar plates and
biochemically characterized by the indole, methyl red, Voges-
Proskauer, citrate tests (IMViC; Bioxon) (48). Biochemical
confirmation of presumptive E. coli isolates was done with the
API 20E test (bioMérieux, Hazelwood, MO). All E. coli isolates
formed typical colonies. FC and E. coli levels were calculated
using the MPN tables described in BAM (48). We isolated one to
three E. coli strains from each E. coli--positive tube of fluorocult
brilliant green bile lactose broth. It is known that the confirmation
of only one colony as E. coli is sufficient to regard that broth tube
as positive for E. coli. These positive tubes were used to calculate
the E. coli concentrations as described in BAM (48). All confirmed
E. coli strains were streaked on tryptic soy agar slants, incubated at
37°C for 24 h, and maintained at 3 to 5°C until they were used for
PCR.

Multiplex PCRs for DEP locus identification. All E. coli
strains isolated and confirmed by biochemical tests were analyzed
using two multiplex PCRs to identify several genetic loci
associated with diarrheagenic E. coli exactly as previously
described (23). Briefly, the first multiplex PCR identifies the
following loci: heat-stable and heat-labile enterotoxins (st and lt)
It has been reported that E. coli (44) isolates with typical biochemical reactions in triple stx (LEE)–negative strains can cause hemolytic-DEPs, and (6).

Salmonella Other stx were isolated from 100, 90, 84, 10, and 4 Salmonella. Other Salmonella eae (35).

The presence of 10 P Salmonella E. coli (31).

~ (27). 611 aggR As we described above, polymerase used in PCRs were from Invitrogen (AccesoLab, was analyzed by following the method described in PATHOTYPES AND, (5) pathotypes, and aap | to human renal endothelial cells IN SPROUTS and % stx with the API 20E test were then serologically

Salmonella E. coli (48).

The presence of Salmonella E. coli (48).

Salmonella isolation and identification. The presence of Salmonella was analyzed by following the method described in BAM (48). Briefly, bags containing sprout samples in lactose broth were incubated for 18 h at 37°C (preenrichment). Aliquots (1 ml) from preenriched subsamples were then enriched in tetrathionate (10 ml; Bioxon) and selenite cystine agar (10 ml; Bioxon) and incubated for a further 24 h at 43°C (tetrathionate) or 37°C (selenite cystine). Both enrichment broths were then streaked onto Hektoen enteric agar (Bioxon), xylose lysine desoxycholate agar (Bioxon), and bismuth sulfite agar (Bioxon) plates and incubated at 37°C for 24 to 48 h. Three typical Salmonella colonies and one atypical colony per plate were inoculated in triple sugar iron agar and lysine iron agar and incubated for 24 h at 35°C (48). The Salmonella isolates with typical biochemical reactions in triple sugar iron agar and lysine iron agar were then biochemically identified with a commercial biochemical kit (API 20E, bio-Mérieux, México D.F., Mexico). Cultures presumptively identified as Salmonella with the API 20E test were then serologically identified using both the somatic polyvalent (O) test and the Salmonella serological flagellar (H) test (O and H antisera were purchased from the Institute of Epidemiological Diagnosis and Reference of the Secretariat of Health, México D.F., Mexico). Salmonella isolates were streaked onto tryptic soy agar, incubated at 35°C for 24 h, and maintained at 3 to 5°C. Salmonella isolates were sent to the Institute of Epidemiological Diagnosis and Reference for serotype identification.

Statistical analysis. The Pearson correlation coefficient was used to compare relationships between the presence of CB, FC, E. coli, DEPs, and Salmonella. A P value of <0.05 was considered significant. Statistical analysis was performed using Statistica (version 8, StatSoft, Inc., Tulsa, OK).

### RESULTS AND DISCUSSION

In the 100 samples analyzed, CB, FC, E. coli, DEPs, and Salmonella were isolated from 100, 90, 84, 10, and 4 samples, respectively (Table 1). The populations of CB ranged from 6.2 to 8.6 log CFU/g. The FC and E. coli concentrations were between <3 and 1,100 MPN/g. The CB levels observed are in agreement with the 7.3 to 8.5 log CFU/g of alfalfa sprouts reported previously (6). In a study done in the United States, FC were isolated from alfalfa sprout samples at concentrations ranging from 1.1 × 103 to 2.4 × 105 MPN/100 g of sprouts (44); Klebsiella pneumoniae isolates were found in most of the FC-positive samples in that study.

The DEPs identified included ETEC, EPEC, and STEC; these were isolated from 2, 3, and 5% of samples, respectively. In these positive samples, the concentrations ranged from 210 to 240 MPN/g for ETEC, 28 to 1,100 MPN/g for EPEC, and 3.6 to 460 MPN/g for STEC. No E. coli O157:H7 isolates were detected in any STEC-positive samples (Table 2). For the STEC, only the stx2 locus was detected in one strain, the stx1 locus was detected in three strains, and both stx1 and stx2 were detected in one strain (Table 2). STEC strains have been identified from raw foods in Mexico, including vegetable salads (5) and carrot juice (46). The pathogenesis of STEC is linked to different virulence factors, such as Shiga toxins (the products of stx1 and stx2) (35). It has been reported that the products of stx1 and stx2 cause different degrees and types of tissue damage (31); the product of stx2 is more toxic than that of stx1 to human renal endothelial cells (31). Other important virulence determinants in STEC include the locus of enterocyte effacement (LEE), which is shared by EPEC. This 35- to 45-kb pathogenicity island is responsible for the formation of attaching and effacing lesions on intestinal epithelial cells (35). It contains the eae gene encoding the outer membrane adhesin intimin, which mediates tight contact between STEC or EPEC and intestinal epithelial cells (27). Although the LEE island is carried by STEC strains, its presence is not essential for pathogenesis, and some eae (LEE)–negative strains can cause hemolytic-uremic syndrome and occasional outbreaks (2, 18, 29, 35, 43). These eae-negative STEC strains are postulated to have

<table>
<thead>
<tr>
<th>Microorganism or indicator</th>
<th>Minimum</th>
<th>Median</th>
<th>Maximum</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB</td>
<td>6.2</td>
<td>6.9</td>
<td>8.6</td>
<td>100</td>
</tr>
<tr>
<td>FC</td>
<td>&lt;3</td>
<td>41</td>
<td>1,100</td>
<td>90</td>
</tr>
<tr>
<td>E. coli</td>
<td>&lt;3</td>
<td>11</td>
<td>1,100</td>
<td>84</td>
</tr>
<tr>
<td>DEPs</td>
<td>&lt;3</td>
<td>&lt;3</td>
<td>1,100</td>
<td>10</td>
</tr>
<tr>
<td>Salmonella</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4</td>
</tr>
</tbody>
</table>

a Minimum, median, and maximum values are in log CFU per gram for coliform bacteria and in most probable number (MPN) per gram for fecal coliforms and E. coli (n = 100 samples). CB, coliform bacteria; FC, fecal coliforms; DEPs, diarrheagenic E. coli pathotypes; ND, not determined.
other putative adherence- and virulence-associated factors, which include STEC agglutinating adhesin (Saa) and subtilase cytotoxin (SubAB) (43). Many STEC strains also produce enterobohemolysis, encoded by the plasmid-borne \( ehxA \) gene, but its role in pathogenesis remains uncertain (11, 17). Considering the uncertainties regarding the role of stx subtypes and putative virulence factors in STEC pathogenesis, it is not clear whether many of the eae-negative STEC strains can cause severe human illness.

EPEC is a leading cause of diarrhea in developed countries (16, 47). Typical EPEC strains contain both eaeA and bfp, while atypical EPEC strains contain only eaeA (26). Unlike typical EPEC strains, which are found only in humans, atypical EPEC strains have been isolated from a variety of animal species (12). ETEC represent another major cause of diarrhea in developing countries and in visitors from regions where ETEC is not endemic (traveler’s diarrhea) (35). ETEC are of human origin and, hence, are indicators of contamination from fecal sources. They are usually transmitted by contaminated food. The ETEC pathotype has been isolated from mung bean sprouts (9) and carrot juice (46).

In this study, \( S.\ enteroxidans \) was detected in 4% of the alfalfa sprout samples and DEPs (ETEC, EPEC, or STEC) in 10% (Table 1). This is in agreement with our previous reports about the isolation of \( S.\ enteroxidans \) (1.1%) in alfalfa sprouts from supermarkets of a different Mexican city (Queretaro City) (6) and with the isolation of \( S.\ enteroxidans \) (5%) and DEPs (10%) from mung bean sprouts in markets of Pachuca City (9). The \( S.\ enteroxidans \) serotypes identified included \( S.\ enteroxidans \) serotype Typhimurium in three samples and \( S.\ enteroxidans \) serotype Enteritidis in one (Table 2). Both STEC and \( S.\ enteroxidans \) Typhimurium were identified in one sample (Table 2). The \( S.\ enteroxidans \) serotypes isolated from sprout samples have been associated with many cases of human illness and have been isolated from different foods in Mexico (24).

Table 2. Diarrheagenic E. coli pathotypes, loci associated with diarrheagenic E. coli, DEP concentrations, and Salmonella serotypes identified in alfalfa sprout samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>DEP/locus (DEP concn [MPN/g])</th>
<th>Salmonella serotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>EPEC/eaeA (120)</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>Non-O157 STEC/stx₁ (460)</td>
<td>—</td>
</tr>
<tr>
<td>12</td>
<td>—</td>
<td>Typhimurium</td>
</tr>
<tr>
<td>19</td>
<td>ETEC/st (210)</td>
<td>—</td>
</tr>
<tr>
<td>27</td>
<td>Non-O157 STEC/stx₂ (3.6)</td>
<td>—</td>
</tr>
<tr>
<td>39</td>
<td>—</td>
<td>Typhimurium</td>
</tr>
<tr>
<td>42</td>
<td>ETEC/st (75)</td>
<td>—</td>
</tr>
<tr>
<td>50</td>
<td>Non-O157 STEC/stx₁ (460)</td>
<td>—</td>
</tr>
<tr>
<td>63</td>
<td>EPEC/eaeA (28)</td>
<td>—</td>
</tr>
<tr>
<td>77</td>
<td>Non-O157 STEC/stx₁ (240)</td>
<td>Typhimurium</td>
</tr>
<tr>
<td>82</td>
<td>Non-O157 STEC/stx₁, stx₂ (11)</td>
<td>—</td>
</tr>
<tr>
<td>89</td>
<td>EPEC/eaeA (1,100)</td>
<td>—</td>
</tr>
<tr>
<td>94</td>
<td>—</td>
<td>Enteritidis</td>
</tr>
</tbody>
</table>

Table 2. Diarrheagenic E. coli pathotypes, loci associated with diarrheagenic E. coli, DEP concentrations, and Salmonella serotypes identified in alfalfa sprout samples

—, not detected; st, thermostable enterotoxin locus; eaeA, intimin locus; stx₁ and stx₂, Shiga toxin 1 and 2 loci.

Bacterial pathogens can contaminate fresh vegetables by primary contamination (while growing and during harvest) or secondary contamination (during washing, slicing, soaking, packaging, and preparation). However, sprout-associated outbreaks have largely been linked to seeds contaminated with pathogenic microorganisms (36) rather than postproduction contamination. Field sources of seed contamination with pathogenic microorganisms include soil, water, wild and domestic animals, drift and runoff from adjacent farms, and manure (1, 38). In addition, microorganisms may contaminate sprouts during packaging, distribution, and sale. An additional concern is the possibility that small producers in Mexico use nonpotable water for sprout production. Cifuentes et al. (10) have reported that households use untreated wastewater, treated wastewater effluent from interconnected reservoirs, or natural rainfall as irrigation water in central Mexico. Also, it is necessary to consider the potential of the DEPs to survive on seeds and grow during the sprouting of seeds. In effect, we have observed that EAEC, non-O157 STEC, EIEC, EPEC, and ETEC strains survived at least 90 days on mung bean seeds at 25 ± 2°C (21). In addition, we have observed that STEC, EIEC, ETEC, and EPEC strains all grew during the germination and sprouting of alfalfa seeds, reaching counts of approximately 5 and 6 log CFU/g after 1 day at 20 and 30°C, respectively (22). However, the fact that DEP and \( E.\ coli \) were recovered at low levels would suggest postharvest contamination of sprouts.

No correlation existed between CB and DEPs or between CB and \( S.\ enteroxidans \). Neither FC nor \( E.\ coli \) correlated with \( S.\ enteroxidans \) in the sprout samples. FC and \( E.\ coli \) have been used as indicators of fecal contamination of food for many years. Nevertheless, the value of the fecal coliform assay as a fecal contamination indicator is nullified when nonfecal-origin bacteria are the principal microbes detected by the assay (37, 39). On the other hand, in this study, \( S.\ enteroxidans \) was isolated in the absence of \( E.\ coli \) in one sprout sample. This coincides with our previous reports about isolation of \( S.\ enteroxidans \) from mung bean sprout samples that tested negative for \( E.\ coli \) (9) and with reports of \( S.\ enteroxidans \) being isolated from different foods that tested negative for \( E.\ coli \) (14, 20, 40, 45). Many researchers agree on the need to expand the search for pathogenic microorganisms beyond just FC and \( E.\ coli \) to include species such as \( S.\ enteroxidans \). Our results suggest that CB, FC, and \( E.\ coli \) are not adequate indicators of the possible presence of \( S.\ enteroxidans \) in alfalfa sprouts.

In addition, the results indicate that alfalfa sprouts in Pachuca, Mexico, pose a potential risk of foodborne illness in the local population and visitors. This because some alfalfa samples were contaminated with \( S.\ enteroxidans \) and DEPs. The \( E.\ coli \) pathotypes found in the alfalfa sprout samples have pathogenic potential. The minimal infective dose is between \( \log^{5} \) and \( \log^{7} \) CFU for ETEC and EPEC and between 1 and 100 CFU for STEC (35). Although the infective dose for ETEC and EPEC is high, this level could be reached in 100 g of some positive alfalfa sprout samples (1,100 MPN per g × 100 g = \( \log^{5} \)) (Table 2). On the other hand, the concentrations of STEC per gram in positive alfalfa sprout samples were within the minimal infective dose (Table 2).
Prevention and mitigation of the risk associated with DEP in sprouts requires the incorporation and consistent application of good agricultural practices and good manufacturing practices throughout the sprout production process, from crop to harvest to retailer (36). Proper sprout handling and processing practices must be promoted and implemented by both Mexican sprout growers and consumers (36).

Finally, although the number of alfalfa sprout samples analyzed in the current study may be considered small, sufficient information was obtained to conclude that the incidence of DEPs and Salmonella in alfalfa sprouts represents a potential public health hazard in Pachuca, Mexico.

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